The Microbial Decomposition of Chromium Tanned Leather

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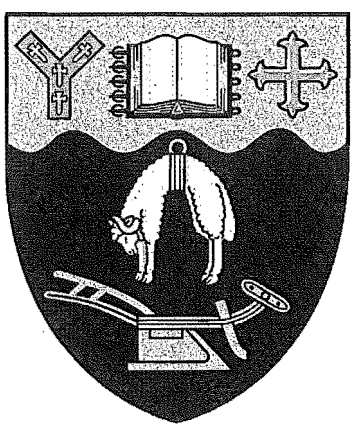

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ABSTRACT

The microbial decomposition of chromium tanned is poorly understood with relatively few reported studies existing. In this study, investigations into the microbial decomposition of leather waste, under a variety of simulated environmental conditions, were performed. Also investigated was the action of collagenase and pepsin enzymes on leather substrates and the effects of chromium oxidation state on proteolytic hydrolysis.

Animal derived waste such as leather, containing high levels of nitrogen (*ca.* 16 %) are generally considered high quality resources capable of degrading readily. However, the tanning process, whereby chromium and pesticides are incorporated into the collagen matrix, results in the reduction of substrate quality and reduced microbial decomposition.

In this study, significant decomposition (20 % as evidenced by nitrogen mineralization), was observed after long term (330 day) incubation. No increase in decomposition was observed with carbon and nitrogen amendments, although hydrolysis (autoclaving) of samples resulted in extensive decomposition (75 %) after an initial 20 day delay.

The cumulative enzyme action of collagenase and pepsin was required for the complete dissolution of chrome leather (95 %), suggesting that collagenolytic activity is required to cleave the collagen structure, prior to proteolysis. Pepsin was not affected by chromium, whereas collagenase was significantly inhibited by concentrations of Cr(III) greater than 1 ppm, when available in solution, but was not inhibited by bound inert chromium in leather.

Results from microbial and enzyme studies suggest that the intracellular physiology of decomposer microorganisms may be affected by the assimilation of chromium bound amino acids, although further work is needed in this area.

CHAPTER 1 : INTRODUCTION

It is estimated that worldwide, more than 600,000 tonnes of chromium containing waste is generated by the leather industry each year (Taylor *et al.*, 1997). Historically, leather waste has been disposed of in landfill sites, but increased restrictions of land disposal have prompted investigation into alternative methods of disposal.

Chromium is added to raw hide during leather manufacture to produce chromium tanned leather. This is done to prevent microbial degradation and produce a more durable product. However, as Cr(III) is a large component of the leather industry waste (4%), the disposal of such material is of increasing environmental concern. Typically, the chromium in the waste, which is primarily in the trivalent form, Cr(III), has a lower biological toxicity than Cr(VI), the other prevalent oxidation state. Issues with respect to the relative toxicity of Cr(III) are the reason increased dumping restrictions have been placed on material enriched in this metal.

In the United States, a conflict currently exists over the toxicity of Cr(III). Although the Environmental Protection Agency (USEPA) regulates the disposal of contaminants, the Resource Conservation and Recovery Act (RCRA) and the Comprehensive Environmental Compensation and Liability Act (CERCLA – 'Superfund Act') conflict over recognition of Cr(III) toxicity. It is agreed by all parties that Cr(VI) is highly toxic, but only the RCRA makes a distinction of Cr toxicity based on oxidation state. Industries legally disposing of Cr(III) contaminated waste under provisions of the RCRA, may be contravening the CERCLA, requiring decontamination of landfill sites at a later time (Rutland, 1991).

The case is less confused in New Zealand with the introduction of the Resource Management Act (RMA) in 1991. Under the RMA, resource consents must be obtained prior to the disposal of any contaminants into the air, land, or water from any place of industry (Scott, 1992). There are no intrinsic declarations made regarding the toxicity of Cr(III) compounds in the RMA.

Consents are granted on a case by case basis, with the nature of the waste, method of disposal, and possible environmental impact assessed (Scott, 1992).

The toxicology of chromium compounds with respect to prokaryotes is poorly understood. However, the chemistry of chromium in mammalian systems is better defined. Under normal mammalian physiological conditions, Cr(VI), the most oxidised form of chromium, is readily taken up into cells through nonspecific ion channels, in a similar manner to phosphate and sulphate (Stearns *et al.*, 1995).

Although Cr(III) compounds rarely cross cellular membranes, studies have shown the accumulation of chromium within cells. Evidence for the accumulation of Cr within cells comes from the use of ^{51}Cr for cell lysis and apoptosis studies (Sanderson, 1982). Conversion of Cr(VI) to Cr(III) traps the ^{51}Cr within the cell, which is released on cell lysis, providing a measure of cell lifetime. This intracellular reduction of Cr(VI) to Cr(III) occurs readily, involving the oxidation of ascorbate, glutathione, and cysteine. Cr(V), Cr(IV), superoxide, singlet oxygen, and hydroxyl radicles are formed in the process (Shumilla *et al.*, 1998). Superoxide and hydroxyl radicles have been implicated in Cr(VI) carcinogenicity and genotoxicity, which have been shown to result from DNA strand breaks, DNA interstrand and DNA-protein crosslinking, and inhibition of DNA replication (Katz and Salem, 1994).

Studies of Cr(III) cellular uptake have also determined that much of the intracellular Cr(III) is associated with the nucleus (Okada *et al.*, 1983), with Cr(III)-DNA adducts found in cells exposed to Cr(VI). It was shown by Shumilla *et al.* (1998) that Cr(III) exhibits a greater affinity for DNA than for amino acid ligands, including cysteine and histidine. In contrast, other heavy metals, including cadmium, mercury, and zinc, elicit cellular toxicity by binding directly to protein sulphydryl groups.

Studies by Kortenkamp *et al.* (1996) on the intracellular conversion of Cr(VI) to Cr(III) by glutathione and ascorbate, showed that chromium toxicity was most likely due to single-strand breaks in DNA, or by modification of the purine and pyrimidine bases. It was found that the reduced Cr intermediates were

responsible for the damage, and not Cr(III). However, toxicity caused by DNA-DNA cross-linking by Cr(III) entities was not ruled out (Okada *et al.*, 1983; Kortenkamp *et al.*, 1996).

Cr(III)-DNA adducts have been shown to increase rates of RNA synthesis, with a corresponding decrease in replication fidelity. This is thought to result from polymerase bypass of existing DNA lesions (Snow, 1994). Thus the presence of Cr(III) within cells may enhance the mutagenicity of an unrelated DNA lesion.

In plants, both Cr(III) and Cr(VI) are taken up, resulting in toxicity (McGrath, 1982), with the prevailing view that Cr(VI) is more toxic than Cr(III) (Peterson and Girling, 1981). Toxicity to plants included reductions in root and shoot growth, chlorosis, and plant death, with the majority of chromium taken up remaining in the root tissue (Skeffington *et al.*, 1976; Lahouti and Peterson, 1979; McGrath, 1982). Chromium was found in the vacuoles and cell walls of roots, with the small proportion in the leaves and cell walls. This was shown to be principally Cr(III) (Sharma *et al.*, 1995).

The inhibition of growth of both roots and shoots of barley seedlings was observed to be greater with Cr(VI) than with Cr(III) (Skeffington *et al.*, 1976). In a similar study (McGrath, 1982) it was shown that at a pH of less than five, the toxicity of Cr(III) was the same as Cr(VI). From this study, it was concluded the Cr(III) toxicity to plants was unlikely, unless in extremely acidic soils (McGrath, 1982).

In a study on the toxicity of chromium tanned leather to maize and tomato, Halligan (1975) showed that the plant growth of seedlings grown in potting mix containing leather waste was reduced and that leaves became chlorotic. The effects were found to last for the first two weeks of growth, but thereafter all plants grew vigorously. At Cr(III) levels as high as 500 ppm, no significant long-term effects on plant growth were observed (Halligan, 1975).

In contrast, relatively little is known about the toxicity of Cr(III) towards microorganisms. Generally, heavy metal toxicity affects bacterial growth, morphology, and biochemistry (Collins and Stotzky, 1989) and is usually via one of three mechanisms: (1) blocking of essential functional groups, (2) displacement of essential ions, (3) modification of active conformation of biological molecules.

Although chromium has been found to play a role in glucose and cholesterol metabolism in mammals, the use of chromium as a catalytic cofactor has not been found in bacterial enzymes. The intrinsic substitution inertness and monovalency (III) in the common biological redox range (-0.4 to +0.8 V), are possible reasons for this (Wackett *et al.*, 1989). Indeed, there are no reports of Cr accumulation within bacterial cells, even though the trivalent ion of Cr(III) is similar in size to both Fe(III) and Al(III), and interacts with a number of ligands strongly. The electronegative phosphoryl groups of lipopolysaccharides (LPS) and phospholipids in bacterial cell walls are also thought to provide sites where strong chromium / cell wall interactions may occur (Ford and Mitchell, 1992).

Cell wall interaction, siderophores, and extracellular polymers account for the majority of tolerance mechanisms used by microorganisms towards chromium. Chromium resistance (or the ability to tolerate) of microorganisms has been observed (Aislabie and Loutit, 1986). Coryneform bacteria isolated from a chromium contaminated marine sediment were able to tolerate 156 μg $Cr(III).ml^{-1}$, with nonmucoid variants exhibiting a lower tolerance. Mucoid strains were found to accumulate more Cr than nonmucoid strains, with greater than 80 % of the metal found in the exopolymer, suggesting that exopolysaccharide-producing bacteria could tolerate higher concentrations of heavy metals, than those that did not produce slime or a capsule.

Decomposition of plant and animal residues has been extensively studied. On addition to soil and with favourable moisture, microorganisms and microfauna will decompose added substrates. Over time substrate quality is

reduced, as the more easily degraded components are metabolised. Water soluble substances and proteins are degraded first, with an accumulation of microbial biomass. A succession of microbial populations decomposes the more recalcitrant components of the substrate, often utilizing existing biomass as a source of readily available nutrients. In the later stages of decomposition when nitrogen is limited, fungi are often the predominant decomposers (Swift *et al.*, 1979). This is due to fungi having a higher C:N ratio than bacteria (Killham, 1994).

Decomposition processes can be monitored by a number of indicators. Weight loss, carbon or nitrogen mineralization, radio-isotope tracking, and the formation of new compounds are examples of methods that have been used previously (Waksman, 1952; de Neve and Hofman, 1996; Tietema and van Dam, 1996; Watkins and Barraclough, 1996).

Under aerobic conditions carbon is mineralized to CO_2, but methane, organic acids, and alcohols are also possible decomposition endproducts under anaerobic conditions. A proportion of substrate carbon may also be lost through leaching of water solubles, or conversion to microbial biomass (Anderson, 1973). Techniques for monitoring the mineralization of organic nitrogen to NH_4^+, NO_3^-, NO_2^- are well developed and give accurate / reproducible results (Bremner, 1965).

Previous studies into the microbial degradation of tanned leather are limited in number. De and Chandra (1979) isolated a nitrogenous waste degrading actinomycete from soil that was capable of solubilizing vegetable tanned leather. Microscopic observations showed that structural changes in leather resulted from microbial decomposition. Datta and Chandra (1982) isolated a single bacterial strain possessing gelatinolytic activity that was able to hydrolyse chromium tanned and vegetable tanned leathers. After incubation for 72 hours at 28-37 °C and pH 7-8, *ca.* 20 % of the chromium tanned substrate had been hydrolysed.

Sivaparvathi *et al.* (1986b) reported that a strain of *Pseudomonas aeruginosa* was able to hydrolyse chrome shavings. However, minimal levels of degradation were observed, unless the sample was autoclaved. This was similarly observed in other studies (e.g. Datta and Chandra, 1982), although in each case the degree of protein hydrolysis was not reported.

Pretreatment of the substrate with acid or alkali was found to have no significant effect on degradation. A pH of 8.5 and incubation at 37 °C were required for extensive decomposition (Sivaparvathi *et al.*, 1986a). A protease from the Pseudomonad was isolated (Sivaparvathi *et al.*, 1986b) that was able to hydrolyse autoclaved leather waste readily after 16 hours incubation.

The decomposition of collagen, the major component of leather has been studied more extensively. Deterioration of raw hides occurs readily and depends on a number of factors: time, temperature, moisture content, and the state of the hide.

The first reported case of an aerobically produced collagenase was from a strain of *Achromobacter iophagus* isolated from cured hides (Welton and Woods, 1973). Collagenase activity by bacteria had previously been reported for *Clostridium* spp., *Bacteriodes* spp. (Gibbons and MacDonald, 1961), and *Staphylococcus aereus* (Waldvogel and Swartz, 1969). The collagenase from *Clostridium histolyticum* has been extensively studied and is the source of most common collagenase used today in the laboratory (Kono, 1968).

In a study of Turkish tanneries, (Birbir and Ilgaz, 1996) reported that the bacterial flora of leather changed during the manufacture of finished leather from raw hide. *Bacillus* spp. were the most prevalent, and were found in nearly all steps of the tanning process. Not surprisingly, *Bacillus* spp., especially *Bacillus cereus* and *Bacillus subtilis* have been correlated with the majority of cases of deterioration of hides and skins (Birbir and Ilgaz, 1996).

Biodeterioration of leather has also been studied in footwear. (Pettit and Abbott, 1975) reported that the conditions in normally enclosed worn shoes,

32 °C, 80-90 % relative humidity, were ideal for the proliferation of microorganisms and possible breakdown of the finished leather. Microbially produced lactate was found to partially solubilize chromium from the leather, opening the leather matrix, and increasing susceptibility of degradation. Microbial growth, especially fungal growth, has been observed on finished leathers after storage in warm humid conditions. However, the damage was thought to occur to the dyes and waxes only, with little evidence for the deterioration of the leather structure itself (Pettit and Abbott, 1975).

Leather is manufactured from the raw hides of cattle and sheep in a multi-step process, producing large quantities of solid and liquid waste. Raw hides are treated with sodium chloride and stored until processing. After soaking and removal of unwanted flesh, the cysteine cross-links in the hair and wool (keratin) are hydrolysed with alkali (generally lime). Sodium sulphide is added as a reducing agent to prevent new disulphide bridges forming. This treatment loosens the hair and wool follicles from the skin, allowing their removal.

Collagen, the major structural component of leather, is a glycine and imino residue rich glycoprotein. The major structural role of collagen results from its molecular conformation. It is comprised of rod-like triple stranded coiled-coil structures, called chains (Figure 1.1).

The structure and stability of collagen is mainly due to interchain hydrogen bonds, involving the occurrence of glycine at every third residue. Stability also results from restricted rotation about the bonds along the polypeptide backbone, due to the high imino residue content. Glycine and imino residues make up 50-60 % of the *ca.* 1000 residues in an alpha chain. These reside in a repeating structure of Gly-X-Y, where *ca.* 25 % of the X and Y residues are proline and hydroxyproline respectively (Brown *et al.*, 1997b). The remainder are residues similar to those found in other proteins. Amino acid side chains are directed towards the outside of the molecule, and play only a minor role in molecular stability.

Cross-links may be formed by a variety of chemical interactions, most notably aldol condensations or schiff base condensations between lysine, hydroxylysine, and their aldehydes. Disulphide bridges between molecules may also form.

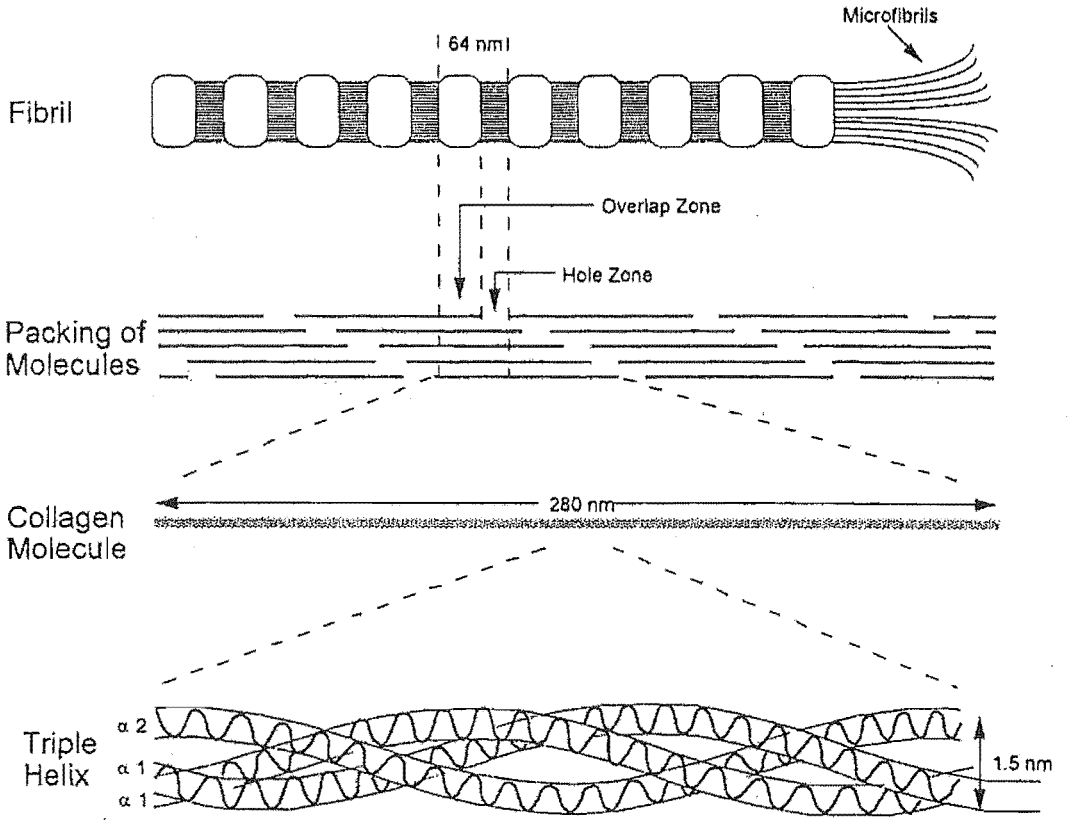


Figure 1.1 : Structure of the collagen fibril. From Shingleton *et al.* (1996).

Fibres making up the collagen structure open up with the alkali treatment, making the structure accessible and permeable to tanning agents. The number of free carboxyl groups is increased and nitrogen is lost as ammonia through modification of guanidine and keto-imide groups in the backbone of collagen chains. This results in a 'swelling' of the fibres, rupturing some of the covalent bonds between adjacent protein chains. The hides are then pickled using

sulphuric acid, prior to tanning with chromium sulphate (Gustavson, 1949; Mann, 1971).

Tanning is the process by which covalent bonds form between reactive amino and carboxyl groups and the tanning agent (Figure 1.2). Stabilization of the collagen complex results in improved resistance to heat, moisture, and microbial degradation. After tanning, the underside of the 'wet blue' leather is 'shaved' to produce leather of appropriate thickness. In this study, the leather waste being examined was from this stage of the process. In New Zealand, a large proportion of the tanned hides is exported overseas as 'wet blue'. Further processing of the hides involves chromium retanning, dyeing, fatliquoring, and finishing.

Fungicides are added to the hides prior to tanning and again after retanning. Phenolic based compounds are gradually being phased out of use, with the introduction of benzothiazoles. These are generally added at 0.05 to 0.10 % of wet hide weight and offer 6 to 9 months protection against microorganisms (DasGupta, 1996; Kennedy, 1996).

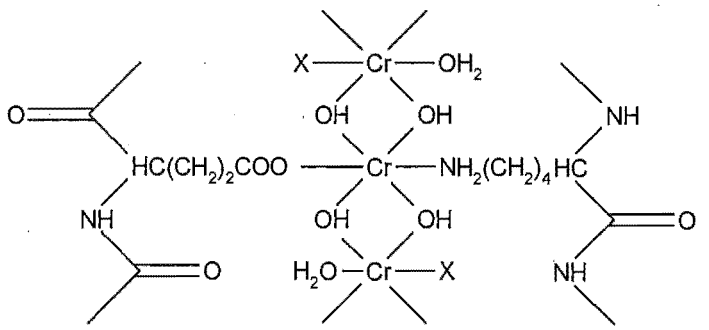


Figure 1.2 : Subsection of the chromium-collagen complex. Modified from Gustavson (1949).

Treatments to dispose of chromium tanned leather waste have existed since the 1920's, when methods were developed for the manufacture of fertilizers from leather waste (Skooglund, 1924). The conversion of waste into animal feed supplements by alkaline and acidic hydrolysis has been extensively

studied, although commercial use of the feed is limited due to high salt and chromium contents (El Boushy *et al.*, 1991; Raju *et al.*, 1997).

Recently, Brown *et al.* (1996) reported on the use of alkaline proteases to hydrolyse leather waste, with recovery of chromium and gelable fractions. The chromium fraction produced has been found suitable for the retanning and fatliquoring stages of leather processing, although some variation in the uptake of the chromium occurs (Glasgow, 1997). Hydrolysed protein from the process, with its high nitrogen content (greater than 15 %) and very low chromium content has potential uses as animal feed or fertilizer, in gel and glue formulations, and in the cosmetics industry (Taylor *et al.*, 1992; Brown *et al.*, 1996; Taylor *et al.*, 1997). However the process is energy intensive and requires alkaline treatment prior to enzymatic hydrolysis.

Sustainable disposal of chromium tanned leather wastes is essential under current RMA legislation and thus an appreciation of the extent of leather decomposition is essential.

Chromium tanned leather decomposition is poorly understood. Microbial decomposition and enzymatic studies in the presence of chromium are limited. In addition, recent controversy regarding the toxicity of chromium in soil systems is still uncertain and poorly understood. This thesis aimed to address these shortfalls, and provide a better understanding of decomposition processes as they pertain to chrome tanned leather, hereafter referred to as leather.

The main aims of this study were to:

- Determine the nitrogen, carbon, and chromium content of water and acetone washed leather and relate this to substrate quality
- Investigate microbial decomposition of leather under varying environmental conditions
- Compare decomposition of leather with addition of carbon and nitrogen supplements
- Investigate enzymatic decomposition of leather with respect to surface area and method of sample preparation
- Investigate effects of leather industry fungicides on microbial decomposition
- Investigate the classes of enzymes thought to be responsible for leather decomposition
- Investigate effect of chromium oxidation state, Cr(III) / Cr(VI), on the rate and extent of leather degradation by enzymes.
- Examine chromium toxicity with respect to seed germination and radicle growth

CHAPTER 2 : METHODS AND MATERIALS

2.1. Collection of samples

2.1.1. Leather samples

Leather samples were donated by Tasman Tanning Co. Ltd and the Leather and Shoe Research Association of New Zealand (LASRA), an experimental tannery / research institute.

Dried shavings from LASRA were used for initial experimentation. Additional samples were obtained from Tasman Tanning Co. Ltd. due to the unknown history of the initial samples from LASRA.

Samples were kept refrigerated, with portions being dried at 43 °C for three days for use in subsequent experiments. All dried samples were kept in sealed polythene bags, at room temperature.

2.1.2. Soil samples

Soil samples (depth 0-5 cm) were collected from the grounds of Canterbury University (Ilam - silt loam, pH 6.4), a semi-alpine forest (Cass – sandy podzol, pH 5.7), and a pine plantation (Bottlelake – sandy soil, pH 6.6). Samples were air-dried for 2 – 4 days, 2 mm sieved, and stored in sealed glass jars.

2.1.3. Other samples

Flower litter specimens (*Quercus ilex* and *Liriodendron tulipifera*) were kindly supplied by Delwyn Walker. Fungal tissue (*Chaetomium globosum*) and pine pollen (*Pinus pinaster*) were donated by Laurie Greenfield.

2.2. Determination of moisture, ash, pH, and total nitrogen of samples

2.2.1. Introduction

Moisture, ash, pH, and total Kjeldahl nitrogen (TKN) were determined for all samples. Results from this initial survey were used in defining parameters of the samples in subsequent experiments, with all results recorded on an ash free or oven dry basis. Double-distilled deionised water was used for all water in experimental work.

2.2.2. Measurement of moisture, ash, and pH

Known amounts of air dry (AD) material (*ca.* 500 mg) were accurately weighed into tared crucibles. Crucibles were placed in a 105 °C oven for 15 h, and cooled in a desiccator prior to reweighing to give the oven-dry (OD) weight. To determine ash content, these same crucibles were ignited in a muffle furnace at 570 °C for 8 h, desiccator cooled and reweighed.

The pH of each sample was measured in a suspension prepared from water and sample (in a 2:1 v/w ratio of solid:liquid). A Solstat EPM-610 combined electrode pH meter was used to measure the pH, after shaking a sample suspension for 30 s and allowing any particulate matter to settle.

2.2.3. Determination of total nitrogen (Kjeldahl)

The total nitrogen content of samples used were determined via the semi-micro Kjeldahl method for total-N, as described by Bremner (1965).

Each sample was placed in a 100 ml Kjeldahl flask together with 1.5 – 2.0 ml concentrated H_2SO_4, and a Hg catalyst tablet. Aqueous samples were acidified with cooling and water evaporated before digestion.

Flasks were heated on low heat on an electric digestion stand in a fume cupboard for 15 minutes, then digested on high heat (*ca.* 400 °C) until samples were colourless (*ca.* 2 hours).

When cool, approximately 20 ml of water was added slowly with cooling to flasks. Flasks were connected to a steam distillation apparatus and 8 to 12 ml of 10 M NaOH containing 40 $g.l^{-1}$ sodium thiosulphate added. Samples were steam distilled as per the method of Bremner (1965), into 5 ml of boric acid indicator. Distillate was titrated against standard 2.5 mM H_2SO_4, with deduction of a control titre for reagent blank.

The amount of nitrogen in each sample was determined and recorded as a percentage of oven dry weight (Bremner, 1965; Equation 2.1).

$$\%\,(Kjeldahl\ N\ in\ sample) = \frac{ml\,(titre - blank\ titre) * 0.07 * 100}{mg\,(OD\ sample\ mass)} \qquad (2.1)$$

2.3. Extraction of leather soluble substances and preparation of washed leather samples

2.3.1. Introduction

Fresh shavings were leached in a number of solvents to extract soluble substances. These extracts were inoculated with soil microorganisms to determine if growth in the presence of the extract was possible.

2.3.2. Extraction of water soluble substances

A known amount (30-40 g) of fresh leather shavings was placed into 1 l Schott bottles, 750 ml of water added and contents shaken for 15 minutes. Water at 25 °C, 80 °C, and 100 °C was used as an eluent. Water soluble substances (WSS) were collected by vacuum filtration through GF/A filter paper (1.6 μm, Whatman) and concentrated using a rotary evaporator. Total nitrogen was determined on aliquots from evaporation after quantitative dilution to 100 ml. Oven dried residues were weighed to determine mass of water insoluble substances (WIS) and water soluble substances (WSS).

2.3.3. Microbial growth in presence of water soluble substances

WSS (from 2.3.2) were diluted to 200 $mg.l^{-1}$ with 30 ml water in 100 ml Erlenmeyer flasks. After addition of 300 μl of soil inoculum, the flasks were covered with thin polythene and incubated with shaking at room temperature for 7 days. Control flasks containing similar contents and 4 drops of chloroform, to prevent microbial growth, were stoppered with rubber bungs and similarly incubated.

Samples from flasks were plated out onto nutrient agar (NA, pH 6.9), potato dextrose agar (PDA, pH 5.5), and NA containing 100 ppm Cr ash (Appendix A.3, pH 6.8) to isolate microorganisms capable of growth in presence of WSS.

2.3.4. Solvent extraction and preparation of washed leather samples

Fresh shavings were shaken in distilled acetone at room temperature overnight. After the extraction procedure, residues were collected by vacuum filtration on GF/A filter paper and dried at 43 °C for 3 days, prior to use in subsequent experiments. Samples were analysed for total nitrogen as before (see 2.2.3).

2.4. Total Oxidisable carbon

In order to calculate C:N ratios for the substrates used in the following experiments, it was necessary to determine total organic carbon content. The method used was that of Walkley-Black as described below (Hesse, 1971).

10 ml of 0.167 M $K_2Cr_2O_7$ and 20 ml of conc. H_2SO_4 containing 15 $g.l^{-1}$ Ag_2SO_4 was rapidly added, with swirling, to 500 ml Erlenmeyer flasks, each containing 50 mg of sample.

After standing for 30 min on an asbestos pad, 200 ml of water and 10 ml of conc. H_3PO_4 was added. When cool, 0.5 ml of 0.16 % (w/v) Barium diphenylamine sulphonate was added and solution titrated against acidified standardised 0.5 $mol.l^{-1}$ ammonium iron (II) sulphate until the emerald green endpoint was reached.

A figure of 40 % total oxidisable carbon for glucose was used to calculate a correction factor (f), to correct for an under estimation of recovery by the method (Hesse, 1971).

$$\%\ oxidisable\ organic\ C\ (corrected) = \frac{(blank\ titre - titre) * 0.003 * 100 * M * f}{g\ (OD\ sample\ mass)} \qquad (2.2)$$

$$\%\ organic\ matter = \%\ oxidisable\ organic\ C * 2 \qquad (2.3)$$

$$M = 0.503\ mol.l^{-1}\ and\ f = 1.02$$

where M is the concentration of the standardised ammonium iron(II) sulphate and f the correction factor.

2.5. Microbial decomposition studies

2.5.1. Introduction

Decomposition experiments using soil microorganisms were conducted using leather and leather substitutes as substrates.

Treatments included temperature cycling, moisture variation, and anaerobic conditions. Substrate quality was tested with respect to carbon and nitrogen limitations. Decomposition experiments were also carried out in the presence of simple or complex substrates, and in the presence of pesticides used by the leather industry. The extent of microbial decomposition was measured by release of mineral nitrogen over time, or by weight loss.

2.5.2. Long term decomposition

100 ml Erlenmeyer flasks containing 5 g of soil or sand (organic matter free), and 100 mg of leather were set up in duplicate for all samples tested. Controls consisting of sand or soil without the addition of leather were also set up. 300 µl of soil inoculum and sufficient water to give 30-40 % moisture (dry weight basis) was added. Samples appeared moist but not wet. Bijou bottles containing 2 ml of 2 M H_2SO_4 were suspended with nichrome wire in the necks of the flasks. Microcosms were covered with polythene and incubated at ambient room temperature for up to 180 days.

Microcosms were aerated and moisture levels checked every 7-10 days. Destructive analysis of microcosms (as per 2.5.2.1) was performed at 30 day intervals to determine N-mineralization over time.

2.5.2.1. Procedure for mineral-N determination

Ammonia-N from acid traps and mineral-N in KCl extracts were combined after analysis to give total mineralized-N for each flask.

Acid traps were removed from microcosms and contents carefully transferred with water into 100 ml Kjeldahl flasks. These were connected to the steam distillation apparatus (as per 2.2.3), 4 ml of 10 M NaOH added and the mixture steam distilled for 2 minutes with distillate collected into boric acid indicator, and titrated against standard 2.5 mM H_2SO_4 (Bremner, 1965).

After removal of acid traps, microcosms were examined visually for the presence of microbial growth and pH determined (as per 2.2.2) after addition of 10 ml of water. 30 ml of 2 M KCl was then added to each flask which were shaken for 2 hours or left standing overnight to extract all mineral-N. 10 ml aliquots were analysed via steam distillation (2.5 min) into boric acid indicator after the addition of 200 mg MgO and 100 mg Devarda's alloy (Bremner, 1965). No nitrate was detected at any time.

Net N-mineralization was calculated after deduction of control titres for reagent blanks and values for zero day incubations.

$$\%\,N\ mineralized = \frac{mg\,(NH_3\ N) + mg\,(mineral\ N)}{mg\,(total\ N\ in\ sample)} * 100 \qquad (2.4)$$

$$\%\ net\ N\ mineralized = \%\ N\ mineralized - \%\ N\ mineralized\ at\ day\ 0 \qquad (2.5)$$

2.5.3. Variable vs. constant temperature decomposition

A long term (330 day) microcosm based experiment was conducted in triplicate. Each flask contained 100 mg ground leather in a sand / soil matrix (as 2.5.2 above). After inoculation and addition of water, the microcosms were incubated in the dark in an insulated air temperature incubator – the temperature of which was 22 °C ± 2 °C. An additional series of flasks, similarly setup, was incubated at a variety of temperatures, with the flasks shifted at intervals to different temperature incubators. Both series of flasks were regularly checked for water content and aerated every 7-10 days.

After incubation, acid traps were analysed for ammonia-N and flasks for mineral-N (as per 2.5.2.1). Deductions were made for net mineralization of soil controls and results presented as a percentage of total nitrogen in samples.

2.5.4. Decomposition of autoclaved leather with variable moisture and temperature

The decomposition of autoclaved leather was studied in a short-term experiment (30-day) to determine if microorganisms were capable of decomposing modified (by mild hydrolysis) leather under a variety of temperature and moisture conditions.

Unwashed shavings were autoclaved (121°C, 25 min wet run) and dried at 43 °C to remove water prior to addition to the microcosms. A portion of the sample was ground (< 1 mm) in a Culatti mill to increase surface area.

Microcosms were set up as in other decomposition experiments (see 2.5.2), with flasks containing *ca.* 100 mg of leather. Duplicate flasks were incubated with 5 and 30 % moisture levels at 5, 10, 20, and 30 °C for 30 days. Following incubation, samples were examined visually for microbial growth and mineralized N was detected as before (2.5.2.1).

2.5.5. Decomposition of hide powder

The decomposition of hide powder (collagen) was studied in a 90 day experiment to determine the extent of microbial decomposition of a substrate similar to leather. The hide powder used was free of pesticides and contained only trace amounts of chromium according to the supplier (Sigma).

Microcosms containing 100 mg of hide powder and 5 g of ignited sand were setup as before (2.5.2) and incubated for 90 days. Mineral-N was analysed at 30 day intervals as per 2.5.2.1.

2.5.6. Anaerobic microbial decomposition

2.5.6.1. Introduction

The decomposition of acetone washed ground shavings was studied under anaerobic conditions to determine if decomposition was more extensive under anaerobic than aerobic conditions.

2.5.6.2. Anaerobic microcosm procedure

Into six flasks containing 5 g of ignited sand, 100 mg of acetone washed ground shavings was added, with 300 µl of soil inoculum, and sufficient water to give 40% water-holding capacity. Controls consisted of microcosms similarly set up, without the leather sample. Acid traps containing 2 ml of 2M H_2SO_4 were suspended in the necks of microcosms.

Into two, 2.5 l anaerobic jars, six microcosms were placed – one containing three controls, and the other three sample flasks.

Anaerobic conditions were created by placing 35 ml of water onto an "Anaerocult A" reagent strip. A reagent strip was then quickly placed in the anaerobic jar, with reactive surface facing the microcosms, and a GasPak Disposable Anaerobic indicator added. Jars were sealed and left at ambient room temperature for 30 days. Three sample flasks and controls were incubated aerobically at ambient room temperature.

After 30 days, the microcosms were removed from anaerobic jars and incubated at room temperature aerobically. Concurrently, similar microcosms incubated under aerobic conditions were placed in the anaerobic jars and incubated at room temperature anaerobically, using fresh Anaerocult A strips and indicators.

Acid traps were analysed for ammonia after 30 days (as per section 2.5.2.1) and replaced with fresh traps. After 60 days, both acid traps and the microcosms were analysed for mineral nitrogen as before. (section 2.5.2.1)

2.5.7. Carbon limitation study

2.5.7.1.Introduction

A short-term (40-day) investigation into the decomposition of leather shavings in the presence of an added simple but available carbon source, glucose, was undertaken. Nitrogen mineralization and weight loss experiments were performed.

2.5.7.2.Microcosm based C limitation study

Microcosms were setup in triplicate, with unwashed shavings or ground shavings in a sand matrix. Glucose was added in soluble form to give ratios of leather : glucose of approximately 1:1, 10:1, and 100:1 (mg per mg basis (OD)). Additionally, Crone's powder (Fred and Waksman, 1928) was added to several microcosms, to determine if mineral salts were limiting with respect to decomposition as evidenced by N-mineralization.

Microcosms were sealed and incubated at ambient room temperature for 40 days, with analysis for mineral-N as before (see 2.5.2.1).

2.5.7.3.Microbial decomposition as measured by weight loss

Clean dry borosilicate glass centrifuge tubes were weighed and 100 mg of unwashed shavings was added to each. Glucose solution was added to give a 1:1 ratio of leather : glucose to several tubes, whilst others had additional water added to maintain moisture levels. 300 µl of soil inoculum was added and tubes capped with thin polythene. Controls for glucose, soil inoculum, and leather with and without the addition of chloroform were also performed. After a 40 day incubation at room temperature, tubes were oven-dried at 105 °C for 15 hours, and cooled in a desiccator, prior to reweighing. Glucose controls were necessary to account for weight loss from the glucose due to partial caramelization of the sugar, caused by thermal degradation at 105 °C.

2.5.8. Nitrogen limitation study

2.5.8.1. Introduction

A short term (40-day) investigation into the decomposition of unwashed leather supplemented with an available nitrogen source (NH_4NO_3) was performed.

2.5.8.2. Microcosm setup for N limitation study

To 100 Erlenmeyer flasks containing 5 g ignited sand, 100 mg of unwashed ground shavings were accurately dispensed. After the addition of 300 µl of soil inoculum, serially diluted NH_4NO_3 solution was added to give a series of carbon : nitrogen ratios from 1:1 to 1000:1. Carbon content of the shavings was determined as oxidisable carbon (see section 2.4), and NH_4NO_3 was counted as two units of available nitrogen.

2.5.9. Decomposition in presence of simple and complex substrates.

Short term (30-day) decomposition experiments were performed to determine if decomposition of an additional substrate was inhibited or enhanced by the presence of chromium tanned leather.

Both unwashed ground shavings and ground autoclaved shavings were decomposed in an ignited sand matrix. An additional substrate was mixed with the leather / sand, to give *ca.* 10 mg of nitrogen in each microcosm – 5 mg from leather, 5 mg from added substrate.

Complex substrates tested were flower litter (*Quercus ilex* and *Liriodendron tulipifera*), fungal tissue (*Chaetomium globosum*), and pine pollen (*Pinus pinaster*). A simple protein substrate, casein, was also used in decomposition experiments. Decomposition of simple and complex substrates was examined in leather-free systems as a control.

Microcosms were inoculated and maintained (40 % moisture) as before (see 2.5.2), and incubated in the dark at room temperature (22 °C). After

incubation, microcosms were examined visually, pH was measured, and both ammonia-N and mineral-N was analysed as before (2.5.2.1).

2.5.10. Decomposition in presence of pesticides used in the leather industry.

2.5.10.1. Introduction

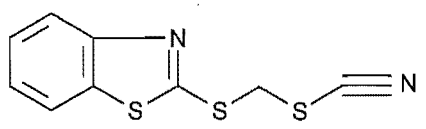
The effect of two commonly used leather industry pesticides on the decomposition of an uncomplexed protein (casein) was examined.

2.5.10.2. Procedure for Pesticide / Casein study

Fifty five mg of casein was weighed into 125-ml flasks containing 5 g of ignited sand. Busan 30LW® (Buckman Laboratories, Figure 2.1) and Preventol WB® (Bayer, Figure 2.2) were added in quantities calculated to give the equivalent industry values for addition to wet hides (0.1 % by weight). Adjustments were made to allow for the substitution of casein as the substrate (Equation 2.6). 300 µl of soil inoculum was added and microcosms adjusted to 30 % water holding capacity. Acid traps containing 2 ml of 2 M H_2SO_4 were placed in flasks, which were sealed with polythene and incubated at room temperature for 15 days. Acid traps and flasks were analysed for ammonia and mineral-N as described previously (2.5.2.1).

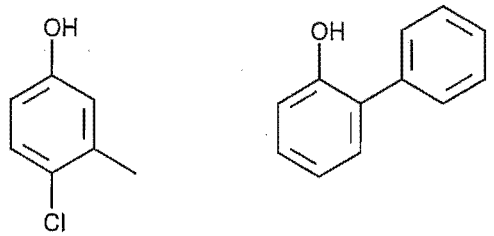
$$equivalent\ leather\ mass\ (wet) = \frac{casein\ mass\ (OD)}{moisture\ content\ of\ leather\ (wet)} \qquad (2.6)$$

Therefore, to simulate application of pesticide to a 100 mg sample of wet hide, 55 mg of casein (AD) requires with 1 $mg.g^{-1}$ of pesticide to give 0.1 % application (w/w).



2-(thiocyanomethylthio)benzothiazole

Figure 2.1 : 2-(thiocyanomethylthio)benzothiazole (30 %), the active ingredient of Busan 30LW (Buckman Laboratories). Unspecified dispersal agents and solvents make up the other 70 % of the pesticide.



4-chloro-3-methylphenol *o*-phenylphenol

Figure 2.2 : 4-chloro-3-methylphenol (35 %) and *o*-phenylphenol (15 %), the two active ingredients of Preventol WB (Bayer). Both are found as sodium salts. The remaining 50 % of the pesticide consists of unspecified dispersal agents and solvents.

2.6. Enzyme degradation studies

2.6.1. Introduction

Enzyme decomposition of a number of samples varying in surface area and method of preparation was examined. Collagenase (Clostridiopeptidase A; EC 3.4.24.3) and then pepsin (Pepsin A; EC 3.4.23.1) were added to samples and incubated for 3 days. The extent of decomposition was observed visually and by a release of soluble N.

The effects of chromium oxidation state were also tested with respect to enzyme activity. Both Cr(III) and Cr(VI) were examined.

2.6.2. Preparation of enzymes

10 mg of Pepsin (Sigma, 3900 units.mg^{-1}) / Collagenase (Sigma, 380 units.mg^{-1}) was weighed into a 1.5 ml eppendorf tube and 1000 µl of buffer added (25 mM HCl (pH 1.7) for pepsin (Windholz, 1976); 0.1 M phosphate buffer (pH 7.4) containing trace amounts of Ca^{2+} for collagenase (Gibbons and MacDonald, 1961; Windholz, 1976). The tubes were shaken gently to dissolve enzyme. After centrifuging, (13,500 rpm for 30 seconds) to remove any undissolved material, supernatants were removed and kept on ice.

2.6.3. Enzyme experimental procedure

Approximately 10 mg of sample was weighed into eppendorf tubes. 100 µl of 10 mg.ml^{-1} pepsin or collagenase supernatant, and 900 µl of buffer solution was added, together with 3 drops of chloroform to prevent microbial growth. Tubes were incubated at 37 °C with occasional inversion. After 72 hours, tubes were removed from the incubator and 2 drops of 1 M NaOH or 1 M HCl added, to adjust the pH to 4-5, the isoelectric point of hide powder. (Gustavson, 1949)

Samples were centrifuged, (10 minutes at 13,500 rpm) and supernatants transferred to Kjeldahl flasks. Pellets were resuspended in the appropriate buffers, taken to isoelectric point, and respun. Supernatants were pooled with those collected prior to pellet resuspension. Approximately 2 ml of pooled supernatant was collected from each sample. 2 ml of conc. H_2SO_4 was added slowly with cooling, and after evaporation of water, a Kjeldahl digestion tablet (Hg) was added. Samples were digested and analysed for total nitrogen as described before. (2.2.3)

2.6.4. Enzyme decomposition with respect to surface area and sample preparation

The effect of surface area on the extent of enzyme activity was examined by using unwashed 2 mm^3 sections, shavings, and ground shavings as substrates. Additionally, the effect of sample preparation, and the order in which the enzymes are used was examined. Autoclaved shavings, acetone washed ground shavings, and water washed ground shavings were also used as substrates.

Hide powder (Sigma) was used to check enzyme efficacy. Eppendorfs containing *ca.* 10 mg of sample were set up (as per 2.6.3), with collagenase. After a 3-day incubation, samples were analysed for total soluble N (as per 2.6.3). Pepsin and HCl were subsequently added to eppendorfs with residues. Soluble N was analysed after another 3-day incubation as before. Controls were established for buffers and samples in buffers.

2.6.5. Effect of Cr(III) and Cr(VI) salts on enzyme action

The effect of chromium oxidation state and concentration was determined using hide powder as a substrate. Chromium sulphate and potassium dichromate were dissolved in water to give stock solutions of 10,000 ppm Cr (on oven dry basis). 1,000, 100, and 10 ppm stock solutions were prepared by serial dilution, with the addition of K_2SO_4 to maintain ionic strength.

Enzyme procedure was as previously described (section 2.6.3), except eppendorfs contained 100 µl of salt solution, 100 µl enzyme, and 800 µl of buffer. Results were normalised to allow for differences in enzyme activity over time, and for sample degradation due to salt solutions.

$$\%\ substrate\ recovered = \frac{mg\ (total\ soluble\ N)}{mg\ (total\ sample\ N)} * 100 \tag{2.7}$$

$$A' = \frac{A*B}{C} - D \tag{2.8}$$

Where the following are percentages:

A′	=	substrate recovery (normalized)
A	=	substrate recovery
B	=	average substrate recovery in 0 ppm Cr salts
C	=	substrate recovery in 0 ppm Cr salts
D	=	substrate recovery in buffer controls

2.7. Electron Microscopy studies

2.7.1. Structural analysis and observation of microbial growth.

Scanning electron microscopy was used to observe physical changes in leather substrates due to decomposition. 'Cold stage', critical point drying and conventional scanning electron microscopy were performed. Samples obtained from decomposition studies were either critical-point dried or viewed on a 'cold stage', so that microbial growth could be observed. Air-drying (43 °C for 3 days) was used for all other samples.

2.7.2. Elemental analysis

The distribution and quantity of Cr in leather samples was determined semi-quantitatively using a SiLi X-ray detector with the electron microscope at high power (20 kV, 400 pA). Analysis of X-ray data obtained was via SEMQuant (Oxford Instruments), calibrated against aluminium.

2.8. Plant Germination studies

The effects of chromium oxidation state were studied with respect to *in vitro* seed germination. To triplicate petri dishes (8 cm) containing filter paper and 3 cucumber seeds, 1.5 ml of 1000 ppm Cr salt solution was added. Moisture levels were maintained by constant weighing. Germination of seeds in water (pH 6.6) was used as a control.

Both Cr (III) – chromium sulphate (pH 3.1), chromium chloride (pH 3.6), and chromium nitrate (pH 3.1); and Cr (VI) salts – potassium dichromate (pH 4.5) and chromium trioxide (pH 1.8), were studied.

After 14 days at room temperature, seedlings were removed from petri dishes and examined. Radicle length was measured and tabulated against chromium oxidation state.

CHAPTER 3 : RESULTS

3.1 Physical and Chemical properties of samples

The amount of total nitrogen (total-N) present in each sample, as a percentage of initial oven-dry weight, shown in Table 3.1, ranged from 14.1 % for ground autoclaved shavings to 15.7 % for water washed ground shavings. Published values for total-N content of collagen are *ca.* 18.6 % (Gustavson, 1949).

The total-N values for the three soils ranged from 0.19 % for Cass, 0.18 % for Bottlelake forest, to 0.58 % for Ilam soil. Nitrogen values for flower litter, pollen, and fungal tissue were in agreement with those found previously (Walker, 1994; Greenfield, 1997). Generally, day zero mineral-N levels were low, with only *Quercus ilex* flower litter containing available levels of mineral-N (2 %).

Total carbon was only determined for a subset of samples. Leather samples were found to contain *ca.* 30-32 % oxidisable carbon, and hide powder 43 %. An estimate of 45 % carbon was used to calculate C:N ratios for litter samples.

Ash contents of leather samples ranged from 5.7 % for water washed leather to 13.9 % for unwashed shavings.

Table 3.1 : Total nitrogen, carbon, mineral-N and ash content of oven dry samples used in this study.

Sample	Total nitrogen (%)	Total carbon (%)	C:N ratio	Mineral-N, % of total N	Ash content, % of oven-dry samples
Unwashed ground shavings	15.1	32.0	2.1	0.4	10.3
Acetone washed ground shavings	15.6	30.3	1.9	0.4	10.0
Water washed ground shavings	15.7	32.0	2.0	0.3	5.7
Autoclaved ground shavings	14.4	ND	ND	0.7	11.4
Hide powder	17.5	43.2	2.5	0.3	0.7
Casein	14.1	31.8	2.3	0.0	4.4
Glucose	0.0	40.0	-	0.0	0.0
Cass soil	0.19	ND	12*	0.3	ND
Ilam soil	0.58	ND	10*	0.4	ND
Bottlelake forest soil	0.18	ND	ND	0.6	ND
Quercus ilex flower litter	1.63	ND	28†	2.3	4.7
Liriodendron tulipifera flower litter	1.76	ND	26†	0.2	5.4
Pinus pinaster pollen	2.59	ND	17†	0.6	3.1
Chaetomium fungal tissue	4.62	ND	10†	1.4	2.9
Busan 30LW fungicide	9.6	ND	ND	0.0	ND
Preventol WB fungicide	0.0	ND	ND	0.0	ND

Note:

- ND : not determined
- Standard error for Total nitrogen did not exceed 0.4 %
- Standard error for Total Carbon did not exceed 1.2 %
- * C:N ratios as per personal communication (Greenfield, 1997)
- † Total carbon of litter samples estimated at 45 %.

3.2. Chemistry and biology of water soluble substances (WSS)

3.2.1 Chemical nature

The amount of water-soluble substances (WSS) obtained from the leather samples is shown in Table 3.2. Hot water extraction of the WSS resulted in the highest yield (8.3 %). Qualitative analysis of WSS extracts using mass spectroscopy (courtesy of Chemistry department, University of Canterbury) showed the presence of TCMTB and phenolic compounds from fungicides used by tanneries.

3.2.2 Microbiological growth in presence of WSS

The extent of microbial growth in the presence of WSS after 7 days incubation is shown in Table 3.2. Most microbial growth was observed with the hot water WSS extracts, with small flocculent fungal growths and turbidity obvious. Both cold and boiling water extracts showed very little turbidity or evidence of microbial growth. Control WSS containing chloroform exhibited no microbial growth.

Observations from 48-hour growth on NA and PDA plates revealed the presence of small white mucoid glistening bacterial colonies. These were also observed on PDA and NA supplemented with 100 ppm Cr ash (Appendix A.3).

Table 3.2 : The amount of water soluble substances (WSS), ash content of water insoluble substances (WIS) from water washed leather, and extent of microbial growth in presence of WSS.

WSS extraction procedure	WSS, % of initial sample mass (ash free)	Ash content of WIS (%)	Microbial growth in 0.2 $g.l^{-1}$ WSS after 7 days
Cold water	2.7	4.7	+
Hot water	8.3	5.0	++
Boiling water	2.9	5.2	+

Note

- Microbial growth observation is in the range 0-++++, where ++++ represents extensive microbial growth.
- Standard error of WSS did not exceed 1.5 %.

3.3. Microbial decomposition studies

3.3.1. Long-term decomposition: Comparison of decomposition at ambient and variable temperature.

Results from long-term (330 day) decomposition of leather shavings at ambient and variable temperature are shown in Table 3.3. An incubation temperature profile is shown in Figure 3.1 for leather decomposed at variable temperature. Higher levels of N-mineralization were found for samples decomposed in a sand matrix (11.4 %), than in soil (1.9 %).

After subtracting controls (Equation 3.1), more mineral-N was recovered from sand microcosms incubated at variable temperature than at constant ambient temperature, 20.0 % and 11.4 % respectively.

Visual evidence of microbial growth and decomposition odour was not observed in any system. However, leather substrates in soil were difficult to distinguish from the medium after incubation. Substrate residues were clearly observed in microcosms containing sand.

The final pHs of microcosms were very similar to the initial pHs regardless of incubation regime and matrix type.

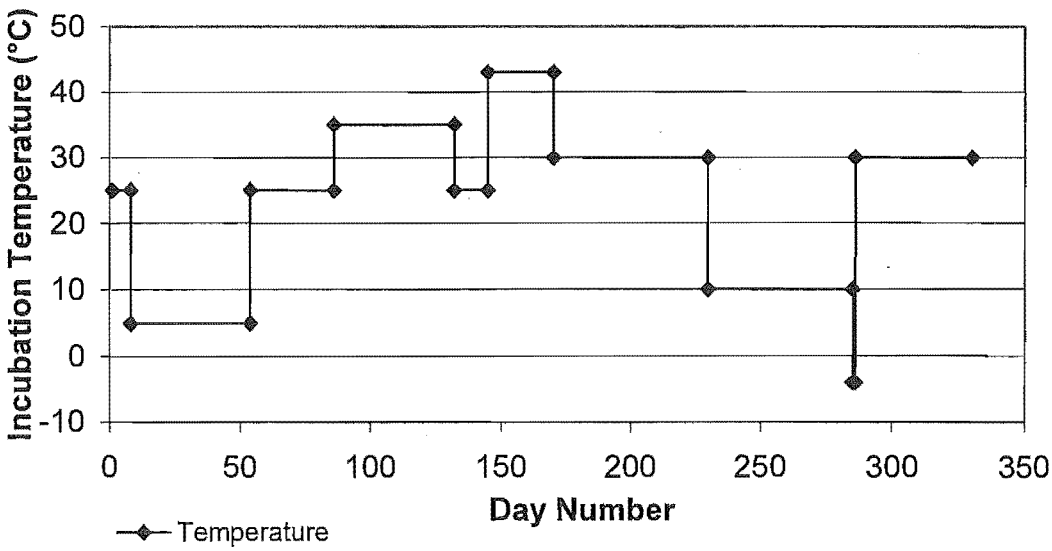
Table 3.3: Total nitrogen mineralized from unwashed leather shavings in sand / soil medium after 330 days incubation, at ambient and variable temperature.

Matrix medium	Temperature regime	Mean leather N-mineralisation (%)†	Final pH
Sand	Constant : 22°C	11.4	6.3
Cass soil		1.9	4.4
Ilam soil		-1.3*	5.4
Bottlelake soil		-2.0*	4.3
Sand	Cyclical : as per fig 3.1	20.0	6.5
Cass soil		4.4	4.4
Ilam soil		-0.8*	5.3
Bottlelake soil		1.6	4.3

Note
- * negative values for N mineralisation show immobilization of released N.
- † mean leather N-mineralization is calculated as per Equation 3.1 below.
- Standard error for mean net leather N-mineralization did not exceed 0.4 %
- Standard error of pH did not exceed 0.2
- Mineralized nitrogen from soil organic matter has been deducted.

$$\%\ N\ mineralization\ of\ leather = \frac{total\ mineralized\ N - mineralized\ soil\ N}{leather\ sample\ N} * 100 \quad (3.1)$$

Figure 3.1 : Temperature profile for microcosm incubation in variable temperature experiment.



3.3.2. Long term decomposition of washed leather

Leather samples washed in acetone or water and then ground to increase surface area, decomposed in a sand matrix, releasing mineral-N. The results from the 180 day incubation at ambient temperature are shown in Table 3.4.

Decomposition of acetone washed samples resulted in the highest recovery of mineral-N after 180 days (3.9 %). Slightly less mineral-N was recovered from Cass and Ilam soil samples (3.3 and 5.4 %). However, more nitrogen was mineralized from unwashed leather samples decomposed in Cass and Ilam soil than in sand (Figure 3.2). Mineralization peaked at 3.8 % for samples in Ilam soil after 120 days, and had decreased after 180 days to 3.5 %. Decomposition in sand based microcosms was minimal, with a maximum of 0.8 % observed after 120 days.

Whereas acetone washed shavings exhibited a steady increase in N-mineralization after an initial 30 day delay (Figure 3.3), both water washed and unwashed shavings only mineralized nitrogen after 90 days.

No microbial growth was detected at any time during the 180 day incubation, but as in section 3.3.1, it was difficult to distinguish leather substrates from soil medium.

pH increased slightly for microcosms containing acetone washed leather, but decreased by 1 pH unit in both Cass and Ilam soils. Other samples showed minimal difference between initial and final pH.

Table 3.4 : Percentage N-mineralization from unwashed and washed ground leather shavings after 90 and 180 day incubations.

Sample treatment	Matrix medium	Day 90 N-mineralized (%)	Day 180 N-mineralized (%)	Initial pH	Day 90 pH	Day 180 pH
Unwashed	Sand	0.18	0.77	7.2	6.7	7.0
	Cass	2.36	3.31	5.4	4.4	4.5
	Ilam	2.50	3.54	6.5	5.5	5.6
Acetone	Sand	2.30	3.88	6.1	6.5	6.8
Water	Sand	0.58	1.53	7.2	6.5	7.3

Note

- Standard error of mineral-N recovery at 90 days did not exceed 0.26 %
- Standard error of mineral-N recovery at 180 days did not exceed 0.40 %
- Standard error of pH did not exceed 0.2

Figure 3.2 : Percentage N-mineralization of unwashed ground leather shavings in sand / soil matrices, over a 180 day incubation.

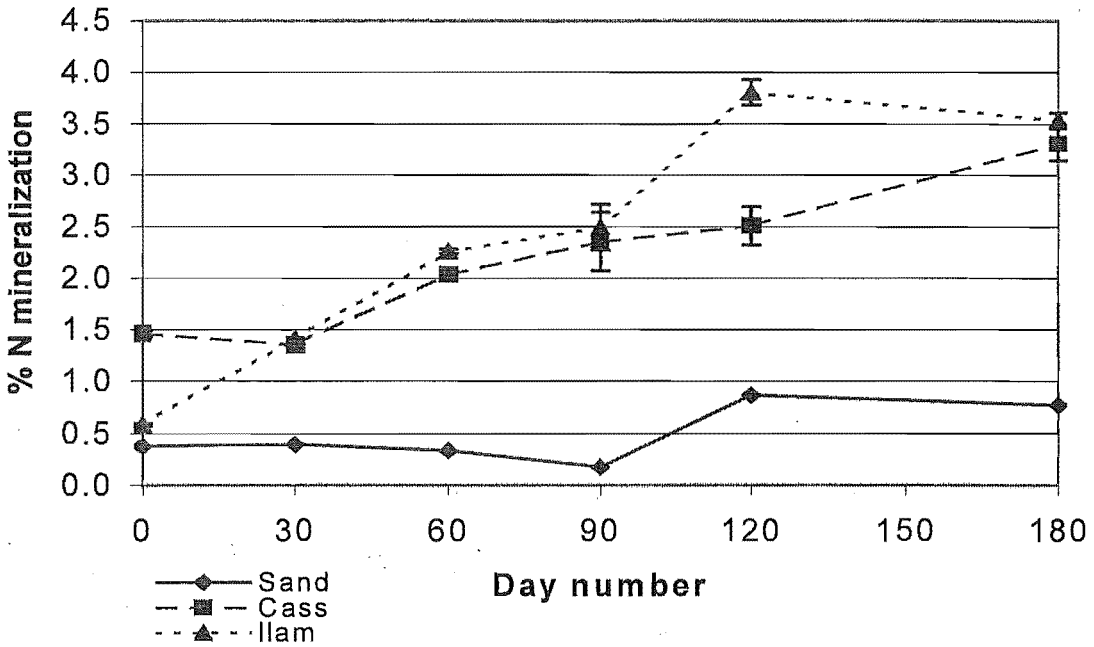
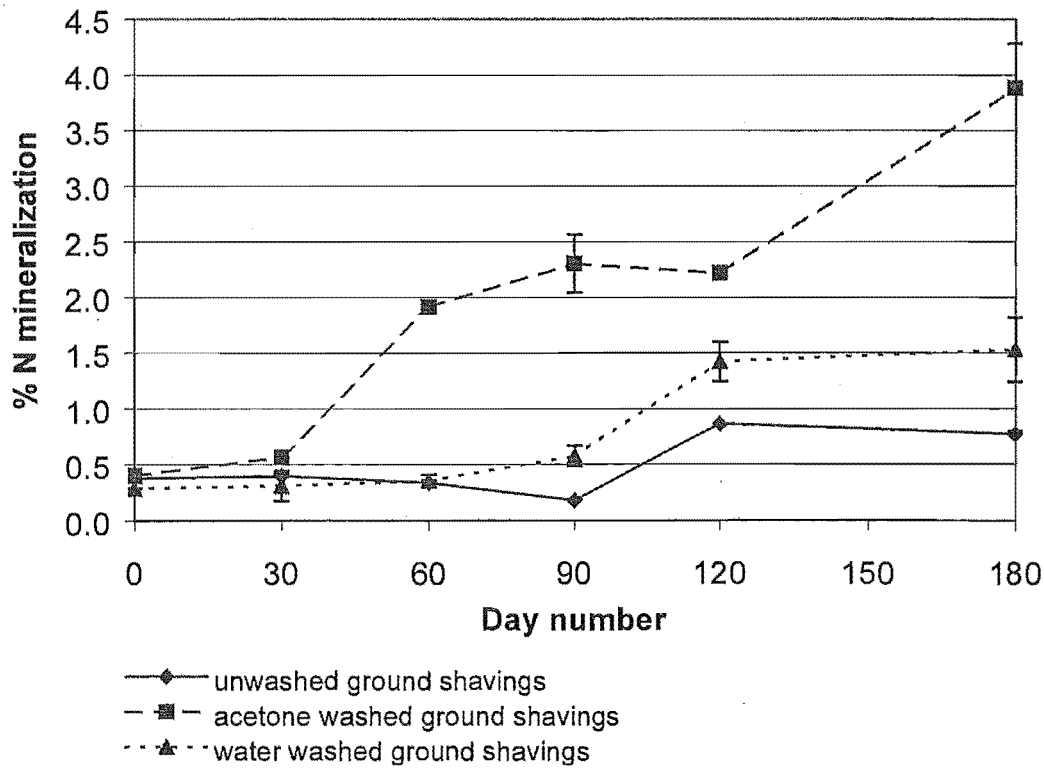


Figure 3.3 : Percentage N-mineralization of ground leather shavings in a sand matrix incubated for 180 days.



3.3.3. Short term decomposition

3.3.3.1. Autoclaved leather decomposition

Microcosm based decomposition of autoclaved leather resulted in a net release of mineral-N. Results for 30 and 60-day incubations are shown in Table 3.5. Full data for the 90-day incubation are shown in Figure 3.4.

Prior to 20 days incubation, both ground and unground autoclaved leather showed minimal decomposition. However, during this time, pH increased from *ca.* 5 to 7.5. Between 20 and 60 days, a great deal of decomposition occurred, with a corresponding increase in KCl extractable mineral-N. After 30 days, both ground and unground leather showed extensive N-mineralization. With further incubation, pH of microcosms varied between 8.4 and 5.9, with final pH *ca.* 7 after 90 days. Bacterial slime and small green fungal crustations were observed after 30 days, accompanied with decomposition odour.

Increasing the surface area of samples by grinding resulted in higher levels of N-mineralization after 30 days compared to unground autoclaved shavings (Figure 3.4). This difference was minimal after 60 days. Standard errors for net mineralization at 30 days were large, with unground material greater than 20 %. This experiment (30 day) was repeated with similar results (Appendix C).

Evidence for selection of Cr tolerant organisms was found, with unidentified isolates of both fungi and bacteria able to grow on NA (pH 6.8) and PDA (pH 5.6) in the presence of ashed leather (72 % chromium by weight) at concentrations as high as 300 ppm (appendix A.3). Fungal isolates from PDA plates were tentatively identified as *Trichoderma* and *Aspergillus* spp. Both Gram positive cocci and Gram negative rods were observed on NA plates, with a large number of filamentous-like chains identified as *Bacillus* sp. At high concentrations of Cr, margins of glistening white bacterial colonies became roughened and non-symmetrical, whilst *Bacillus* colony growth was stunted. Plating colonies back onto non-Cr supplemented media, resulted in the return of normal colony morphology.

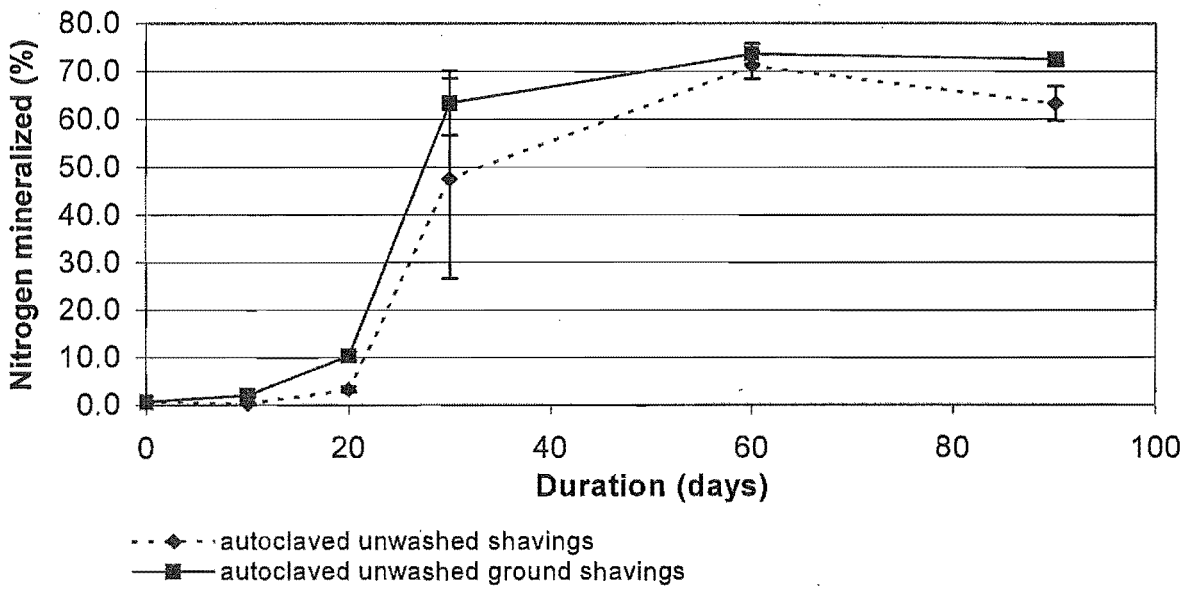
Table 3.5: Percentage total nitrogen mineralized from autoclaved leather, after 30 and 60 days incubation in a sand matrix at room temperature.

Sample	Mean N-mineralization (Day 30 %)	Mean N-mineralization (Day 60 %)	Initial pH	d30 pH	d60 pH
Autoclaved unwashed shavings	47.6	71.2	4.9	8.4	7.2
Autoclaved unwashed ground shavings	63.4	73.6	5.4	8.4	5.9

Note

- Standard error of mean net nitrogen mineralization did not exceed 6.6 %, except day 30 autoclaved shavings, which was 21.0 %.

Figure 3.4 : Percentage mean N-mineralization of autoclaved leather, in a sand matrix over time.



- Graph represents mean ± standard error

3.3.3.2. Decomposition of hide powder

Commercial chromium-free hide powder (1 mm^2 pieces) decomposed readily in a sand matrix, with no appreciable difference in the extent of decomposition after 30 days (Table 3.6). Fungal growth was not observed on substrate, but a decomposition odour was present after 10 days. Substrate became transparent and semi-gelatinous, suggesting presence of a bacterial slime.

Table 3.6 : Decomposition of hide powder in sand as evidenced by N-mineralization.

Incubation period	Mean N-mineralization (%)	Final pH
30 days	53.3	8.7
60 days	46.0	8.4
90 days	48.6	8.1

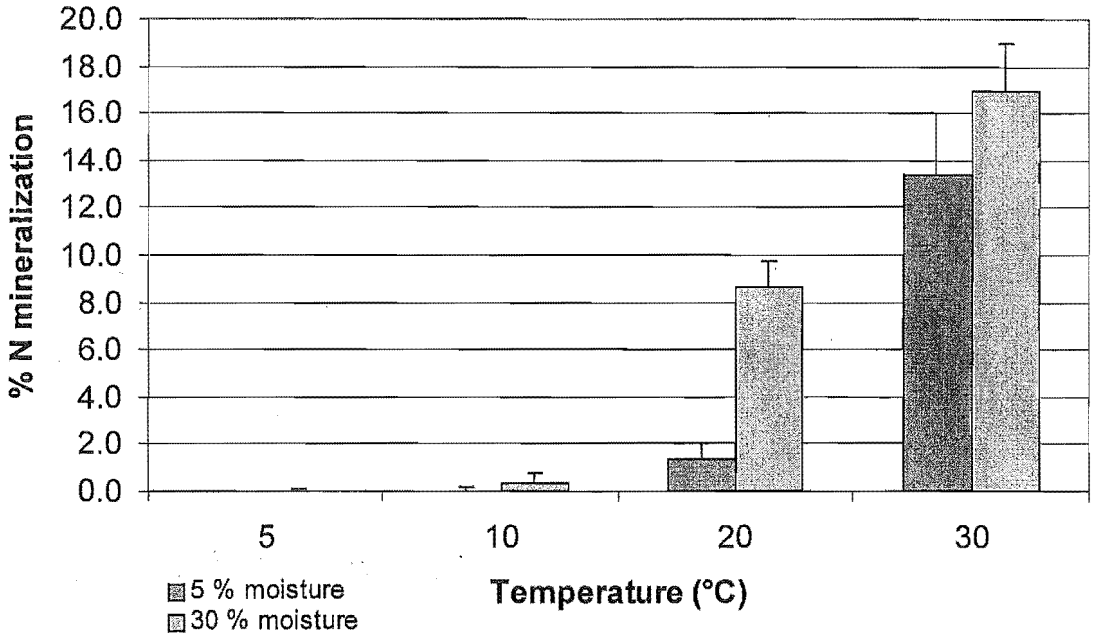
Note
- Standard error of mean N-mineralization did not exceed 19.4 %
- Standard error of final pH did not exceed 0.2
- Result is % of sample-N corrected for mineral-N at day 0.

3.3.3.3. Decomposition with respect to temperature and moisture

The extent of decomposition of ground autoclaved leather shavings was dependent on the temperature of incubation and available moisture. At low incubation temperatures (5 and 10 °C), N-mineralization was minimal, irrespective of moisture content (Figure 3.5). In contrast, N-mineralization at 20 °C was highly dependent on moisture content with *ca.* 5x more N-mineralized at 30 % than 5 % moisture. The greatest amount of decomposition (17 %) occurred at 30 °C, where the effect of moisture content was limited.

The average pH after incubation ranged from 6.1 to 7.9, with the exception of microcosms at 5 °C and 5 % moisture, where the average pH was found to be 3.7.

Figure 3.5 : Mean N-mineralization of autoclaved ground shavings decomposed at various temperatures at 5 and 30 % moisture.



Note

- Standard error for mean net N-mineralization did not exceed 2.6 %

3.3.3.4. Anaerobic decomposition

The 60-day decomposition study of acetone washed leather resulted in a marginal increase in release of mineral-N for samples incubated aerobically and then anaerobically (Table 3.7).

No sign of microbial decomposition of the substrate was observed, either visually, or by odour. Microcosm pH, prior to KCl extraction, was found to have increased slightly for aerobic samples, and decreased for anaerobic samples.

KCl extracts for incubations in both conditions were found to be slightly turbid, suggesting some microbial growth (control microcosms were clear). Samples incubated aerobically then anaerobically were slightly discoloured and the surface of the extract had a grease-like sheen. The nature of this is

unknown, although it is speculated that it may result from breakdown or release of fatty acids from the leather samples.

Table 3.7 : Anaerobic / aerobic decomposition of acetone washed ground leather as evidenced by net nitrogen mineralization

Conditions	Mean Nitrogen mineralization (%)	Initial pH	Final pH
Anaerobic then aerobic	0.09	7.4	8.2
Aerobic then anaerobic	0.43	7.4	6.4

Note
- Standard error for anaerobic / aerobic decomposition did not exceed 0.05%
- Standard error for aerobic / anaerobic decomposition did not exceed 0.11%
- Samples were incubated in each condition for 30 days at 22±2 °C and 30 % moisture.

3.3.3.5.Decomposition in the presence of pesticides

Decomposition of casein was greatly inhibited by the addition of pesticide. Both Busan 30LW and Preventol WB exhibited extensive antimicrobial activity. Mean N-mineralization for the 15-day incubation is shown in Table 3.8. At pesticide concentrations equivalent to those specified by the manufacturers, 1000 $\mu g.g^{-1}$ (pesticide to wet hide weight), complete inhibition of decomposition resulted. Pesticide concentrations of 10 $\mu g.g^{-1}$ resulted in the production of moderate amounts of mineralized-N, with *ca.* 45 % and 60 % for Busan and Preventol respectively (Figure 3.6).

With reduced concentrations (less than 1 $\mu g.g^{-1}$), no inhibition of decomposition was evident – approximately 77 % of total nitrogen was mineralized to NH_4-N.

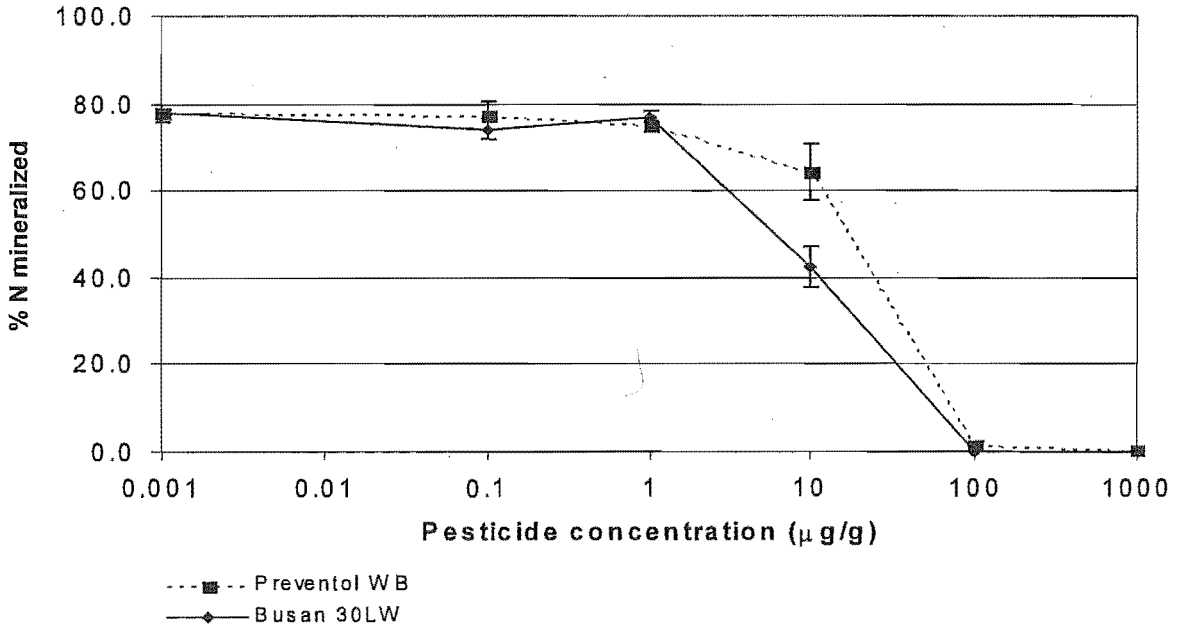
Table 3.8 : Short term (15 day) decomposition of casein in presence of pesticide in a sand matrix.

Pesticide	Concentration ($\mu g.g^{-1}$)	Casein nitrogen mineralized after 15 days	pH initial	pH final
Busan 30LW	**1000**	**0.0**	**8.1**	**7.5**
	100	0.0	ND	7.8
	10	42.4	ND	8.8
	1	77.2	ND	9.0
	0.1	74.2	ND	9.0
	Nil	77.9	7.8	8.9
Preventol WB	**1000**	**0.2**	**10.8**	**9.8**
	100	1.3	ND	8.9
	10	64.3	ND	9.0
	1	75.0	ND	9.0
	0.1	77.3	ND	8.9
	Nil	77.9	7.8	8.9

Note:

- ND: not determined
- Standard error of nitrogen mineralised in presence of Busan 30 did not exceed 4.6%
- Standard error of nitrogen mineralised in presence of Preventol WB did not exceed 6.5%
- 1000 $\mu g.g^{-1}$ was standard working concentration of both Busan 30LW and Preventol WB

Figure 3.6 : Nitrogen mineralization as a percentage of total casein nitrogen, following 15 day decomposition of casein in presence of pesticide, at room temperature with 30 % moisture.



Note

- Extent of mineralization in absence of pesticide is shown at 0.001 $\mu g.g^{-1}$.

3.3.4. Simple and complex substrate decomposition in the presence of leather

Nitrogen mineralization in the presence of simple and complex substrates was assayed, to determine if the presence of leather or leather constituents inhibited microbial decomposition.

The results from the short-term (30-day) decomposition study are shown in Table 3.9. Net mineralization of nitrogen was found to occur for all samples, except flower litter (Figure 3.7).

The extent of N-mineralization occurring in the presence of unwashed leather was lower than for autoclaved leather. Casein exhibited the greatest degree of nitrogen mineralization, whilst both autoclaved shavings and *Chaetomium* fungal tissue mineralized substantial amounts of nitrogen.

Visual observation of microcosms suggests that microbial growth was not inhibited by the presence of the leather, with all samples of the same type exhibiting similar degrees of growth. The degree of aggregate formation between the substrate and sand matrix was highest in microcosms containing unwashed leather and the additional substrate.

Quantification of the proportion of leather-N mineralized from total-N could not be obtained. Attempts to calculate this proportion (Equation 3.2) were based on the assumption that non-leather substrates mineralized nitrogen similarly with and without the addition of leather. However, a qualitative examination of leather-N mineralization could be performed by plotting total-N mineralization as a percentage of total leather-N (Figure 3.8). Mineralization of leather-N was *ca.* 2 to 3x greater with autoclaved unwashed ground shavings than unwashed ground shavings.

$$\textit{leather N mineralized} = \textit{total mineralized N} - \textit{mineralized nonleather N} \qquad (3.2)$$

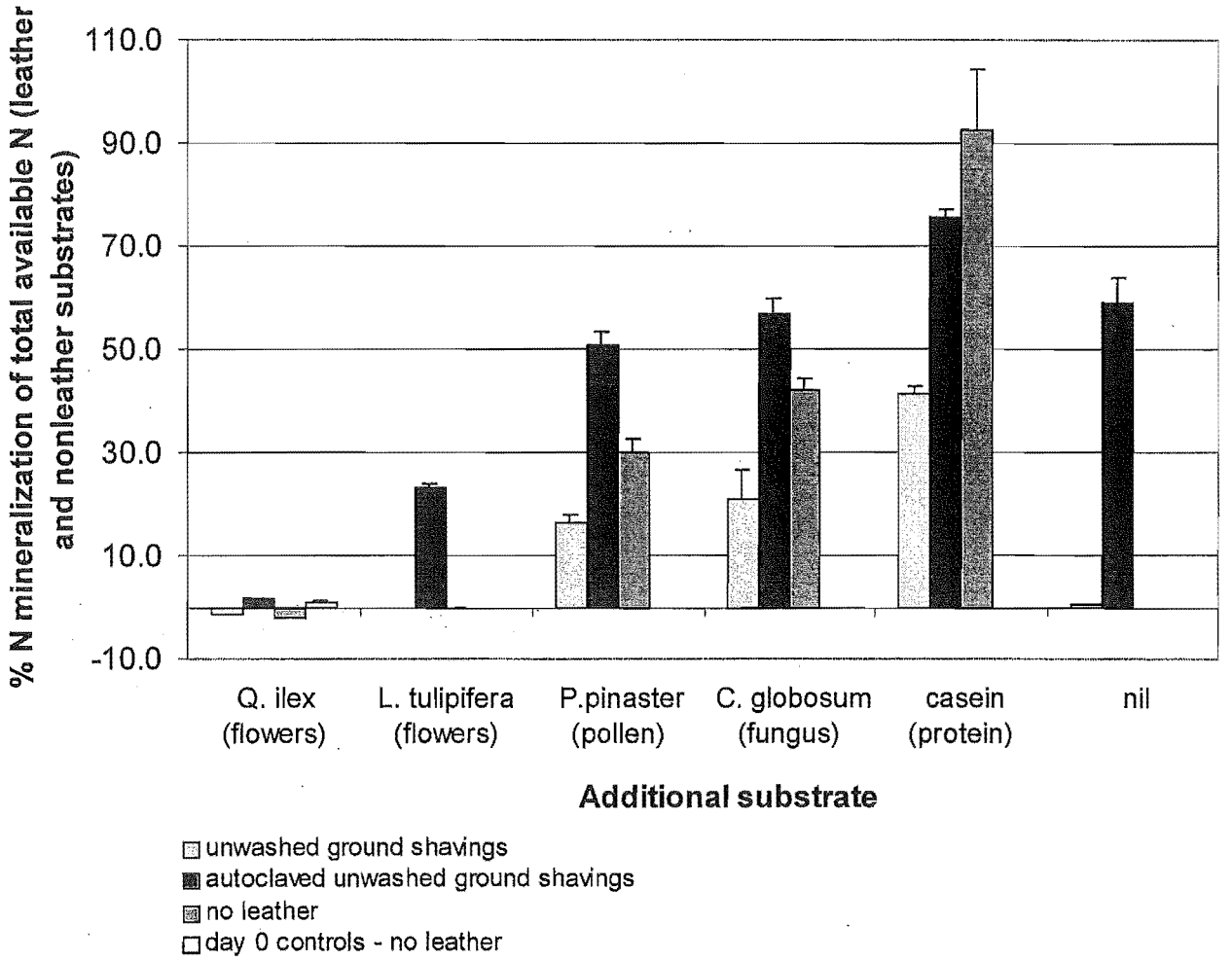
Table 3.9 : Short term decomposition (30 day) of additional substrates in presence of leather.

Leather type	Additional substrate	Microbial growth	Mean N mineralisation (%)	Initial pH	Final pH
Unwashed	*Q. ilex*	++++	-1.3*	ND	6.9
Ground	*L. tulipifera*	++++	-0.2*	ND	7.8
Shavings	*P. pinaster*	+	16.5	ND	7.1
	C. globosum	+++	21.0	ND	7.4
	Casein	0	41.2	ND	7.9
	-	0	0.7	7.2	6.1
Autoclaved	*Q. ilex*	++++	1.8	ND	7.0
Unwashed	*L. tulipifera*	++++	23.2	ND	9.0
Ground	*P. pinaster*	+++	50.7	ND	7.6
Shavings	*C. globosum*	+++	56.9	ND	7.1
	Casein	0	75.5	ND	6.4
	-	0	59.0	7.3	6.3
Nil	*Q. ilex*	++++	-2.1*	4.7	7.1
	L. tulipifera	++++	-0.4*	5.6	8.5
	P. pinaster	++	30.0	6.2	7.7
	C. globosum	+++	41.9	5.7	8.0
	Casein	+	92.5	7.8	7.6

Note

- * negative values for N mineralisation show N-immobilization.
- Standard error for mixed substrate systems did not exceed 5.8 %
- Standard error for single substrate systems did not exceed 11.9 %
- ND : not determined

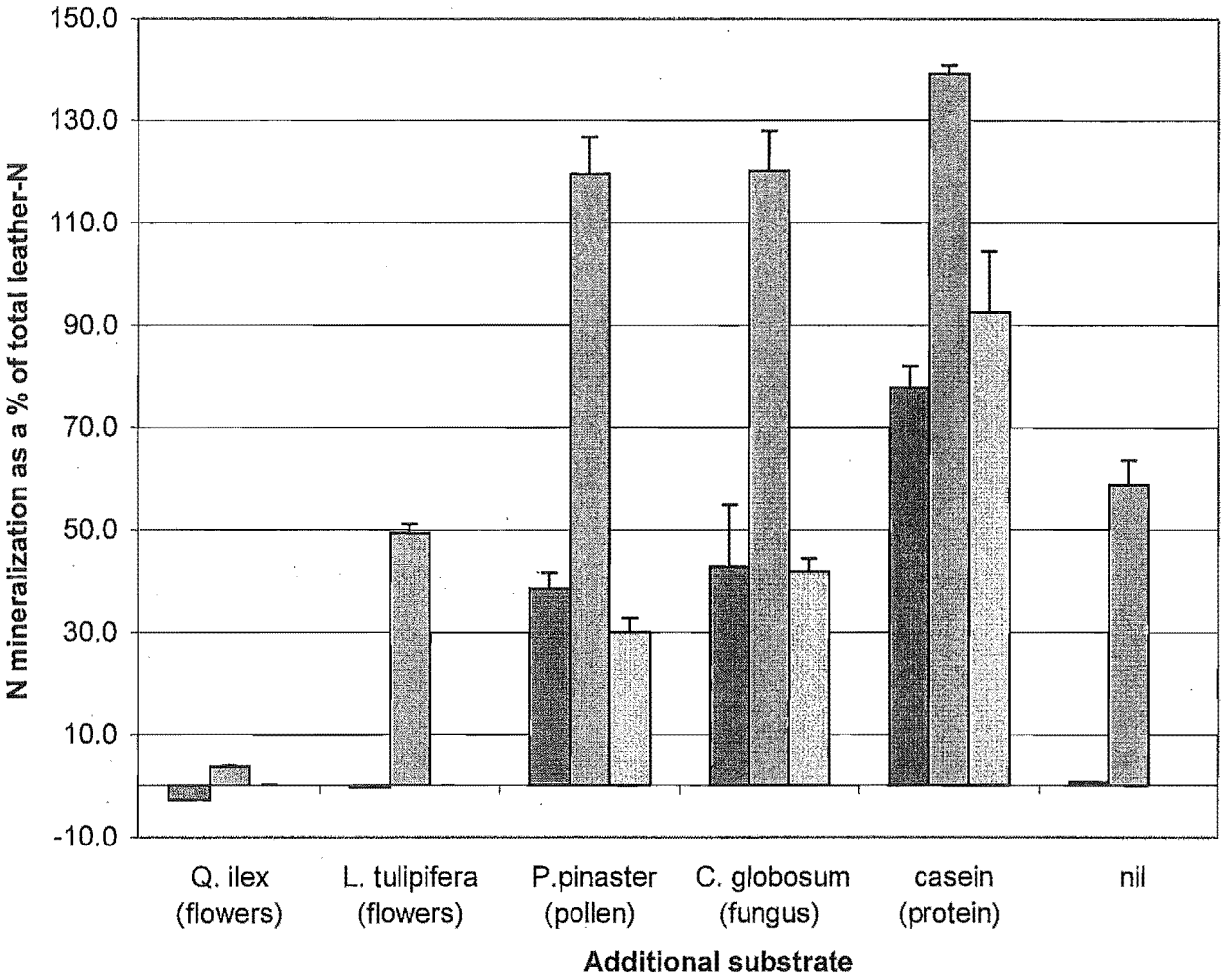
Figure 3.7 : Short-term (30 day) decomposition of mixed leather and nonleather substrates, as evidenced by N-mineralization.



Note

- Graph represents mean ± standard error
- Standard error for all samples did not exceed 11.9 %.

Figure 3.8 : Mean N-mineralization in mixed leather and nonleather systems, at room temperature and 30 % moisture.



Note

- Standard error for all samples did not exceed 7.9 %
- Percentage N recovery is calculated from (mass N recovered)/(total N available from leather).
- Nil leather is shown for comparative purposes.

3.3.5. Leather decomposition with supplemental addition of carbon and nitrogen

3.3.5.1. Decomposition with supplemental carbon and mineral salts

Decomposition of unwashed leather supplemented with carbon, in the form of glucose, did not result in increased N-mineralization. Results for the 40-day decomposition study are shown in Table 3.10.

Although no N-mineralization was detected, microcosms containing 1000 $mg.g^{-1}$ glucose exhibited extensive microbial growth. The blue/grey colour of the leather substrate changed to a dirty green/grey (Figure 3.9) and black sporing structures were evident. Less microbial growth was observed in microcosms containing 10 and 100 $mg.g^{-1}$ glucose.

No increase in N-mineralization resulted from the addition of Crone's powder, a nitrogen free medium containing soluble phosphorous, calcium, and magnesium (Appendix B).

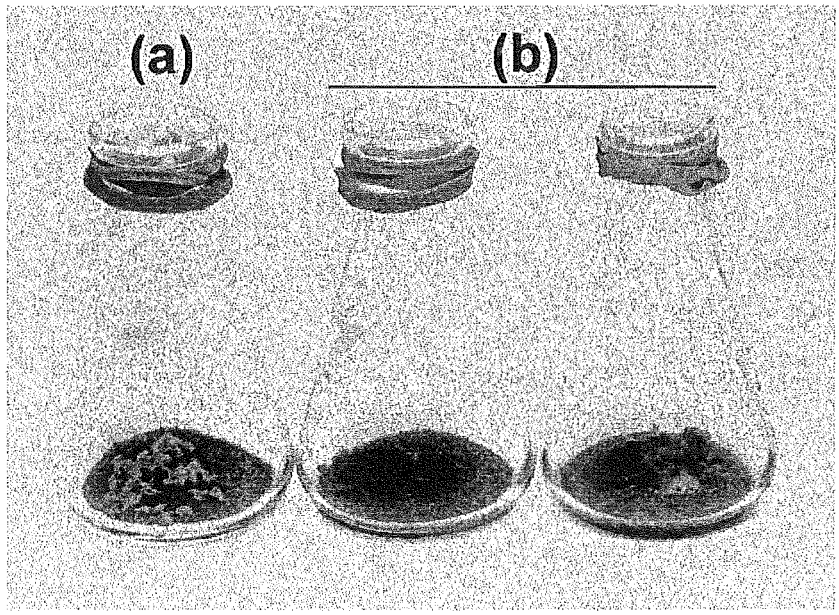
The initial and final pHs of microcosms were similar – *ca.* 5.5 with Crone's powder, and 6.7 to 7.1 without. A decrease pH (1 to 2 units) was observed for microcosms containing high levels of glucose (1000 $mg.g^{-1}$)

Table 3.10 : Mean net mineralization of nitrogen from leather decomposition supplemented with glucose and Crone's powder.

Added glucose (mg per g of leather)	Addition of Crone's powder	Microbial growth	Mean N-mineralization (%)	Final pH
1000	+	+++	-0.3*	4.9
100	+	+	-0.3*	7.1
10	+	+	-0.3*	6.7
0	+	0	-0.2*	6.7
1000	-	+++	0.0	4.6
100	-	+	-0.4*	5.5
10	-	+	-0.4*	5.5
0	-	0	-0.3*	5.6

Note
- * negative values indicate N-immobilization.
- standard error of final pH did not exceed 0.2

Figure 3.9 : Decomposition of unwashed leather with addition of 1000 $mg.g^{-1}$ glucose.



(a) unwashed leather control, (b) extent of microbial growth on unwashed leather shavings with 1000 leather with 1000 $mg.g^{-1}$ glucose.

3.3.5.1.Decomposition with supplemental nitrogen

Microcosms containing unwashed leather with addition of available nitrogen, NH_4NO_3, exhibited minimal microbial growth and mineralization of leather-N (Table 3.11).

Overall, nitrogen mineralization was low, with a maximum of 9.8 % observed for a 5:1 C:N ratio. Microcosms containing high C:N ratios (1:1 and 2:1) showed N-immobilization or incomplete recovery of the NH_4NO_3 nitrogen. These results assume that the recovery of NH_4NO_3 nitrogen was the same as the 1.4 mg of NH_4NO_3 nitrogen in the nonfumigated control (57 %).

The final pH in each system ranged from 4.6 to 5.3, dropping from an initial pH of 6.2.

Table 3.11 : Extent of microbial growth and net nitrogen mineralization from soluble-N supplemented microcosms.

Carbon : Nitrogen ratio in microcosm	Microbial growth	Mean leather-N mineralization (%)	Final pH
1000:1	0	0.0	5.3
100:1	+	0.5	4.9
50:1	+	1.1	5.2
10:1	0	5.8	4.8
5:1	0	9.8	4.9
2:1	+	-19.7*	4.7
1:1	+	-35.3	4.6
No added N	0	0.1	4.8

Note

- * negative values for N-mineralization represent nitrogen immobilization, or differential recovery of added available nitrogen with respect to C:N ratio.
- Recovery of added NH_4NO_3 is assumed to be the same in all microcosms.
- N blank consisted of 1.4 mg soluble N in a sand matrix incubated as per other microcosms.
- Absorption of added soluble-N onto matrix was assumed negligible at day 0.
- Standard error of N-mineralization did not exceed 2.5 %
- Standard error of final pH did not exceed 0.1

3.3.5.2. Decomposition in a carbon supplemented system as observed by weight loss.

Decomposition of leather samples in a sand free system resulted in a loss of weight for samples incubated with a source of available carbon, glucose (Table 3.12).

Carbon supplemented leather samples lost 12.8 to 14.3 % of total mass. A 12 % loss in weight was observed for the glucose solution used to supplement the leather substrates.

Visible changes in glucose amended substrates were observed. Samples took on a reddish hue and become slightly transparent / gelatinous. A faint sweet odour was also detected.

Table 3.12 : Mean net weight loss from glucose supplemented leather in a matrix free system (30 day incubation at room temperature).

Leather sample with glucose	Microbial growth	Mean weight loss (%)
Unwashed shavings	0	14.3
Autoclaved unwashed shavings	+	12.8

Note
- Standard error of mean net weight loss did not exceed 1.6 %
- Corrections have been made for weight loss by controls.

3.4. Enzymatic decomposition studies

3.4.1. Decomposition with single and dual enzymes.

Leather substrates degraded with a single enzyme solubilized significantly less nitrogen than those degraded with sequential enzyme addition (Figure 3.10).

Ground leather was more extensively degraded in both single and dual enzyme systems, with three to five times more N-solubilization observed for ground leather samples than 2 mm cubes of leather.

Hide powder was extensively degraded (> 90 %) by collagenase, but not by pepsin (15 %).

More solubilization of nitrogen was achieved when substrates were treated with pepsin following collagenase action, than when collagenase action preceded pepsin (Figure 3.10).

Following results from this investigation, further enzyme degradation studies were performed in which collagenase action preceded pepsin.

3.4.2. Effect of surface area and sample preparation on enzymatic degradation of leather.

Leather enzymatically degraded with collagenase and pepsin solubilized significant amounts of nitrogen. A surface area effect was observed for unwashed ample with both unwashed shavings and unwashed ground shavings (85 %) solubilizing twice the amount of leather-N as unwashed 2 mm^3 pieces of leather (43 %). (Figure 3.11).

Water washed ground shavings and acetone washed ground shavings solubilized similar quantities of nitrogen as unwashed ground shavings.

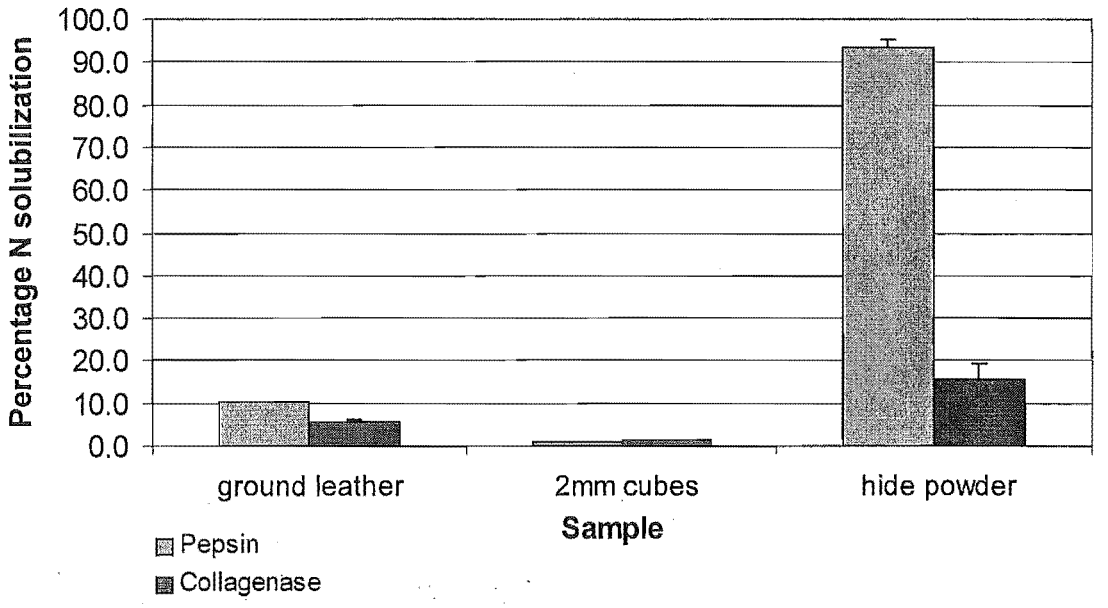
Autoclaved unwashed shavings were more susceptible to enzyme action than unwashed shavings, suggesting possible denaturation and hydrolytic cleavage of proteins by autoclave.

The recovery of nitrogen when hide powder alone was subjected to enzyme action was greater than 95 %.

In controls consisting of buffers (0.025 M HCl or 0.1 PB) and leather without enzyme, it was found that levels of nitrogen soluble at pH 4.5 ranged from 3 % for 2 mm^3 pieces to 85 % for autoclaved leather.

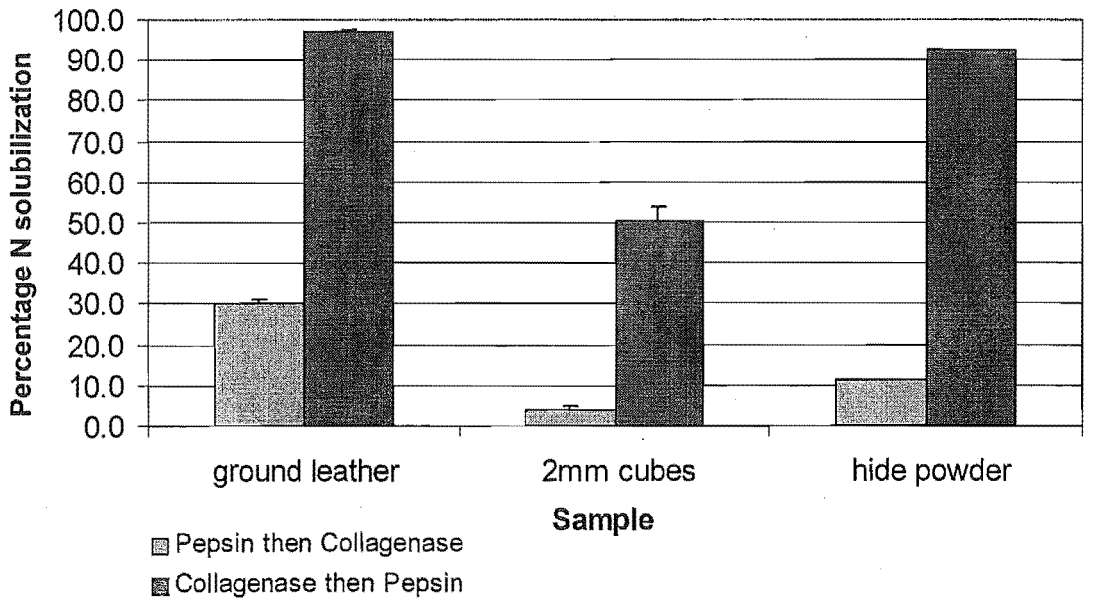
Figure 3.10 : Effect of enzyme sequence on leather degradation.

Single enzyme



(a)

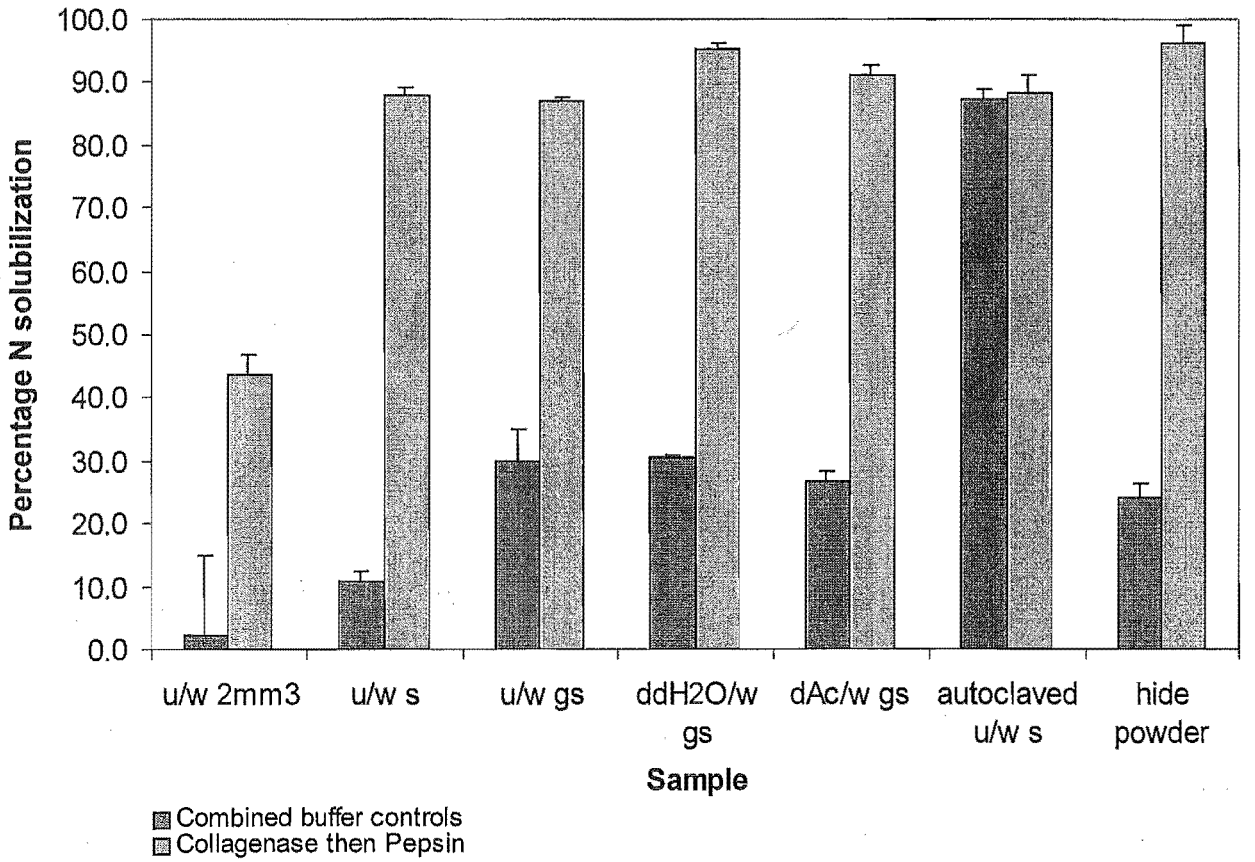
Dual enzymes



(b)

(a) Enzyme action on leather with either pepsin or collagenase; **(b)** Enzyme action with dual enzymes – pepsin/collagenase and collagenase/pepsin.

Figure 3.11 : Enzyme action on leather with respect to sample preparation and surface area.



Notes

- Abbreviations

u/w 2mm3	unwashed 2 mm^3 pieces
u/w s	unwashed shavings
u/w gs	unwashed ground shavings
ddH2O/w gs	water washed ground shavings
dAc/w gs	acetone washed ground shavings
autoclaved u/w s	autoclaved unwashed shavings

- Standard error of percentage nitrogen solubilization did not exceed 12 %

3.4.3 Effect of Cr oxidation state and concentration on enzyme activity

Hide powder was readily degraded in the presence of Cr(III) and Cr(VI) salts, by pepsin and collagenase. Levels of substrate solubilization and recovery were greater than 85 % after allowing for substrate buffer dissolution (Figure 3.12).

As in section 3.4.1, hide powder solubilization by pepsin (75-85 %) was significantly greater than that achieved by collagenase (*ca.* 40 %).

Collagenase solubilized 40-50 % of hide powder irrespective of Cr(VI) concentration. The predominant form of Cr(VI) at this pH (~7) would have been the relatively inert CrO_4^{2-}. With Cr(III), a logarithmic decrease in hide powder solubilization was observed for Cr(III) concentrations greater than 1 ppm. Collagenase activity at 100 ppm Cr(III) was minimal, and completely inhibited at 1000 ppm Cr(III). At pH 7, Cr(III) would have existed as $Cr(OH)^{2+}$ (Table 3.13).

Solubilization of hide powder by pepsin in the presence of Cr(III) (pH 2) was not reduced by tested Cr(III) concentrations (up to 1000 ppm), with *ca.* 75-85 % solubilization observed. At Cr(VI) concentrations greater than 100 ppm, a reduction in hide powder dissolution was observed, with complete inhibition at 1000 ppm Cr(VI). In the pH 2 buffer used, the predominant forms of Cr(III) and Cr(VI) would have been $Cr(H_2O)_6^{3+}$ and $HCrO_4^-$ respectively.

Not more than 15 % of nitrogen was solubilized in enzyme free controls, with more dissolution observed in the presence of 25 mM HCl than the 0.1 M phosphate buffer.

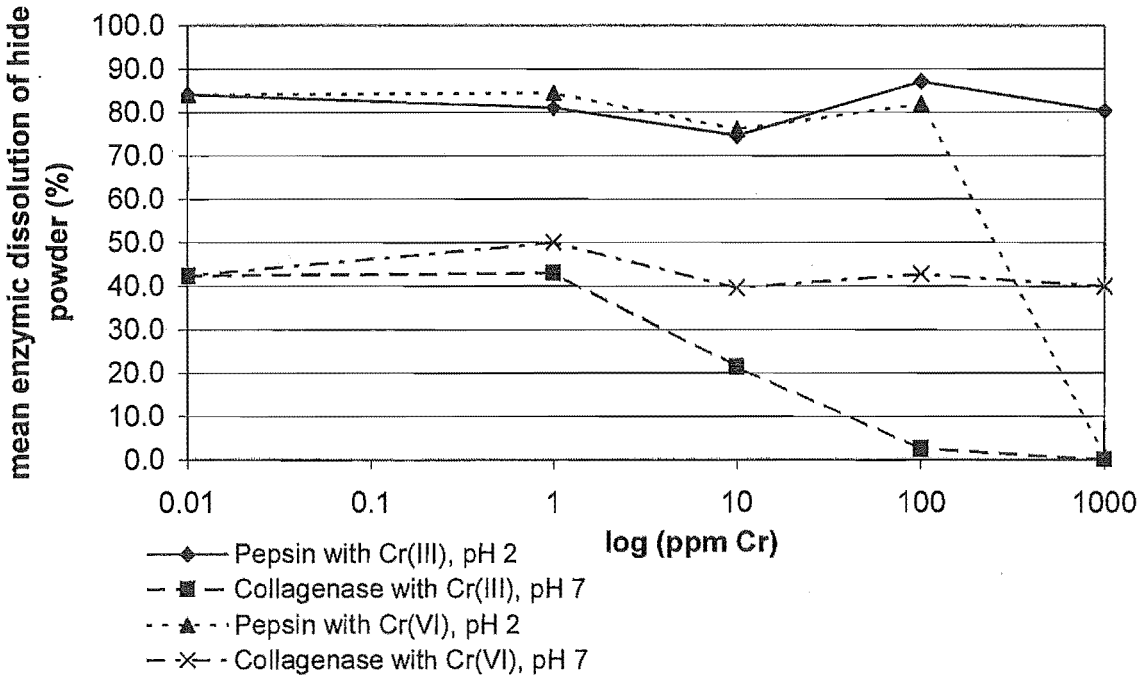
Table 3.13 : Oxidation state and pH of chromium salts in HCl and phosphate buffer.

Cr salt concentration (ppm)	Cr oxidation state	pH in HCl	pH in Phosphate Buffer
1000	III	1.9	6.9
100	III	2.0	7.4
10	III	2.0	7.4
1	III	2.0	7.4
1000	VI	1.9	6.9
100	VI	2.1	7.3
10	VI	2.1	7.4
1	VI	2.1	7.4
0	-	2.1	7.4

Note

- Cr(III) was expected to exist as $Cr(H_2O)_6^{3+}$ in 25 mM HCl buffer and as $Cr(OH)^{2+}$ in 0.1 M phosphate buffer
- Cr(VI) was expected to exist as $HCrO_4^-$ in HCl buffer and as CrO_4^{2-} in phosphate buffer.

Figure 3.12 : Solubilization of hide powder by pepsin / collagenase in the presence of chromium (III) and chromium (VI) salts.



Note

- Results for chromium free systems are shown with a Cr concentration of 0.01 ppm.

3.5. Electron microscopy studies

3.5.1. Effect of sample preparation on physical structure

Physical characteristics of leather samples used in decomposition experiments were found to reflect the method of sample preparation used. Scanning electron micrographs of leather substrates prepared in different ways are shown in Figure 3.13.

Examination of unwashed ground leather (Figure 3.13 (a)) revealed bundles of interconnected collagen fibres packed to form sheets and strands. Structural changes in leather were observed after washing in acetone, followed by grinding (Figure 3.13 (b)). Extensive separation of fibres from bundles and individual fibrils were observed. The resulting increase in surface area is more likely attributable to the acetone treatment than grinding, as both (a) and (b) were ground. The removal of some organic component (possibly pesticide or fatty acid) or lessening of interfibril hydrogen bonding may be the underlying cause. This is supported by the observation that the physical structure of water washed ground leather (not shown) is not significantly different from unwashed ground leather.

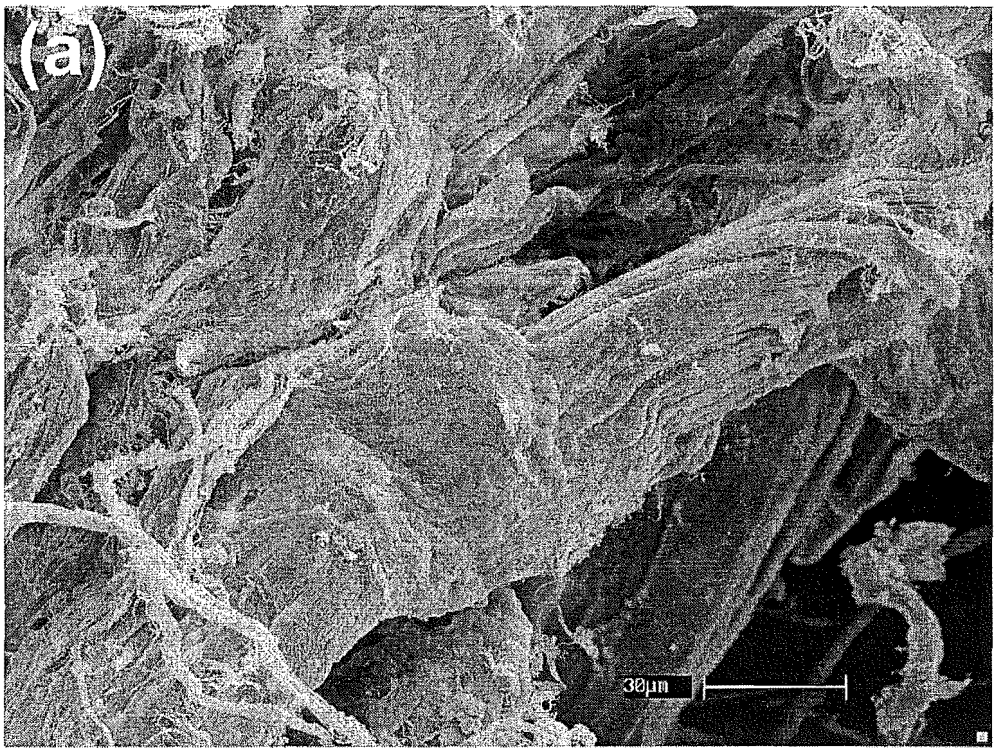
Large structural changes were observed when leather was autoclaved (Figure 3.13 (c)). The basic structure of Figure 3.13 (a) was no longer apparent. In its place, a combination of smooth and highly pitted material was found. Small crystalline star-shaped structures were evident, embedded in the pitted matrix. These were suggestive of salt crystals, although elemental analysis (via SEM) of these structures was inconclusive. It is also possible that they were the remnants of shattered fibrils.

Closer examination (Figure 3.13 (d)) revealed that the pitted surface of the substrate was highly irregular and that the smooth sections were glasslike. No individual fibres or bundles were observed.

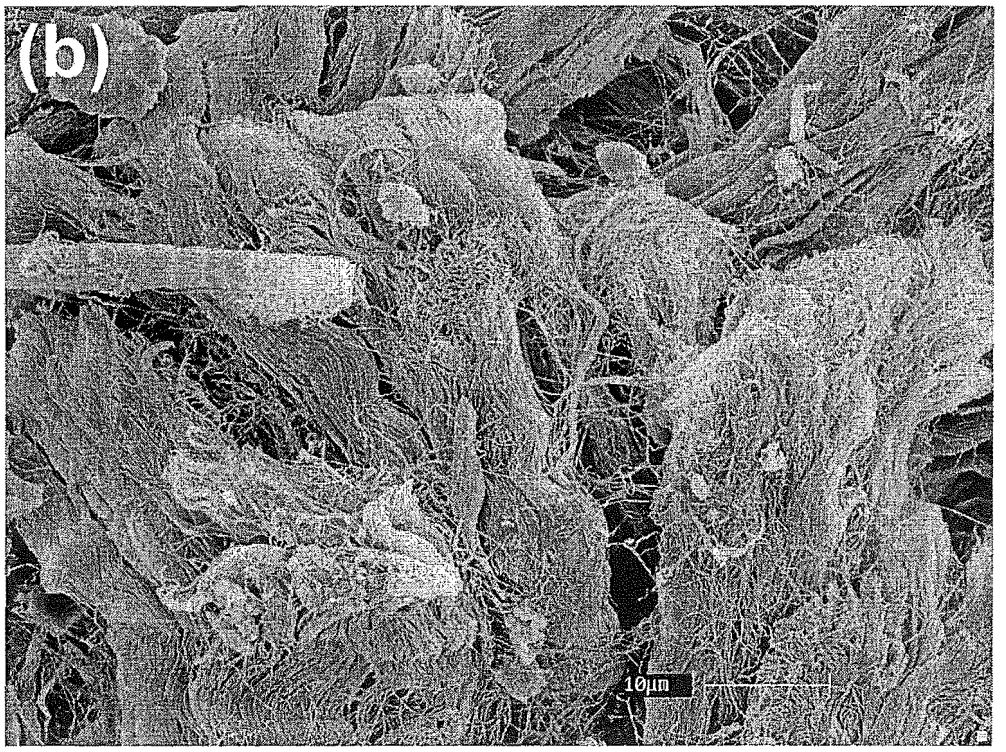
Gross morphological observation of leather leached in 0.025 M HCl for 56 days (Figure 3.13 (e)) revealed structural differences. Collagen bundles and sheets making up the majority of the structure had become smooth and amorphous. Interconnecting fibres and small bundles were obvious, and appeared to be holding the structure together. Further observation at higher magnification (Figure 3.13 (f)) revealed that mild acid hydrolysis of the leather resulted in a loss of coherent structure, possibly due to swelling.

Collagen bundles in hide powder were tightly bound in sheets, with helical bundling of collagen fibres (Figure 3.13 (g)). The structure appeared relatively smooth at low magnification, with a small amount of loose debris visible. Examination of surface detail (Figure 3.13 (h)), revealed a roughened surface, similar to that observed in Figure 3.13 (d).

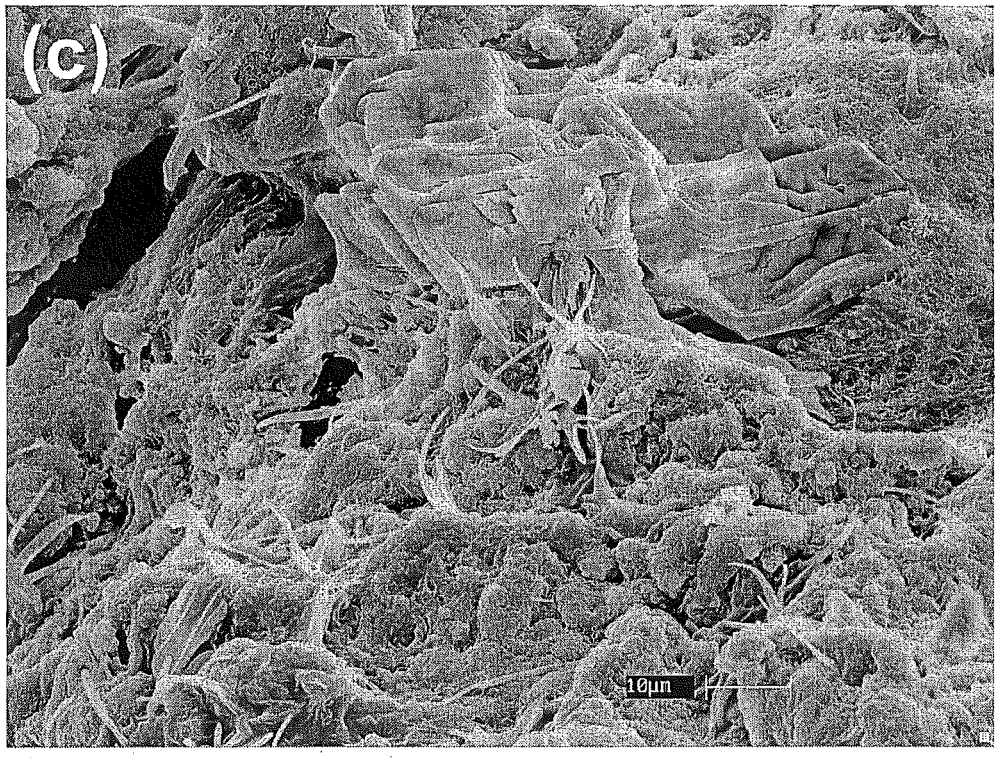
Figure 3.13 : Scanning electron micrographs of leather samples used in decomposition experiments.



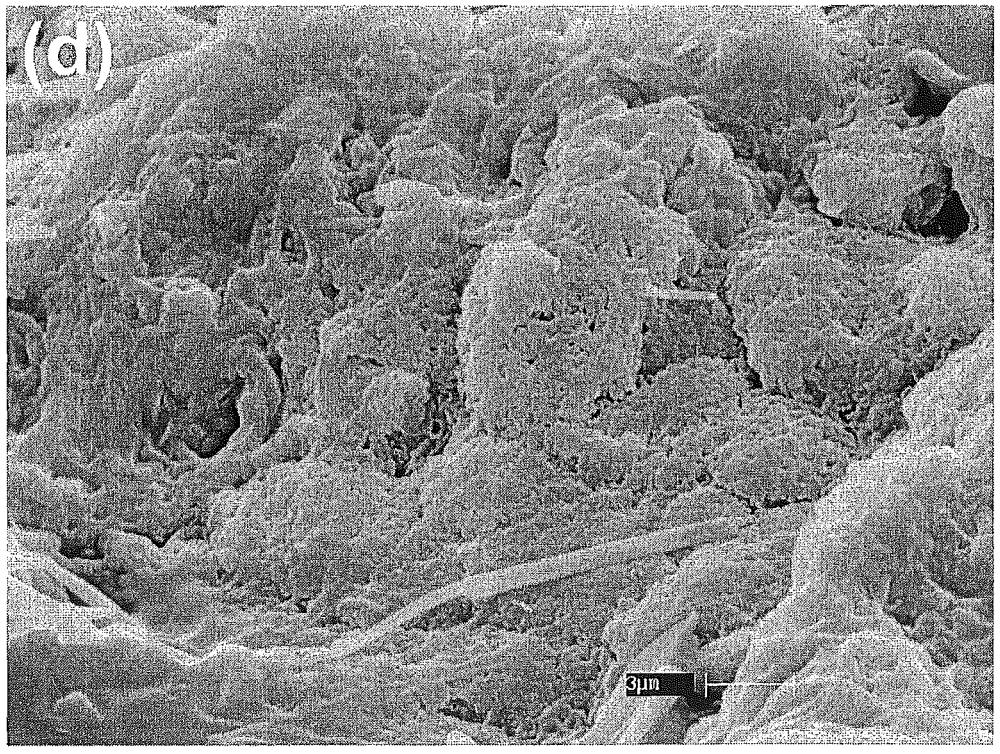
(a) Unwashed ground leather shavings



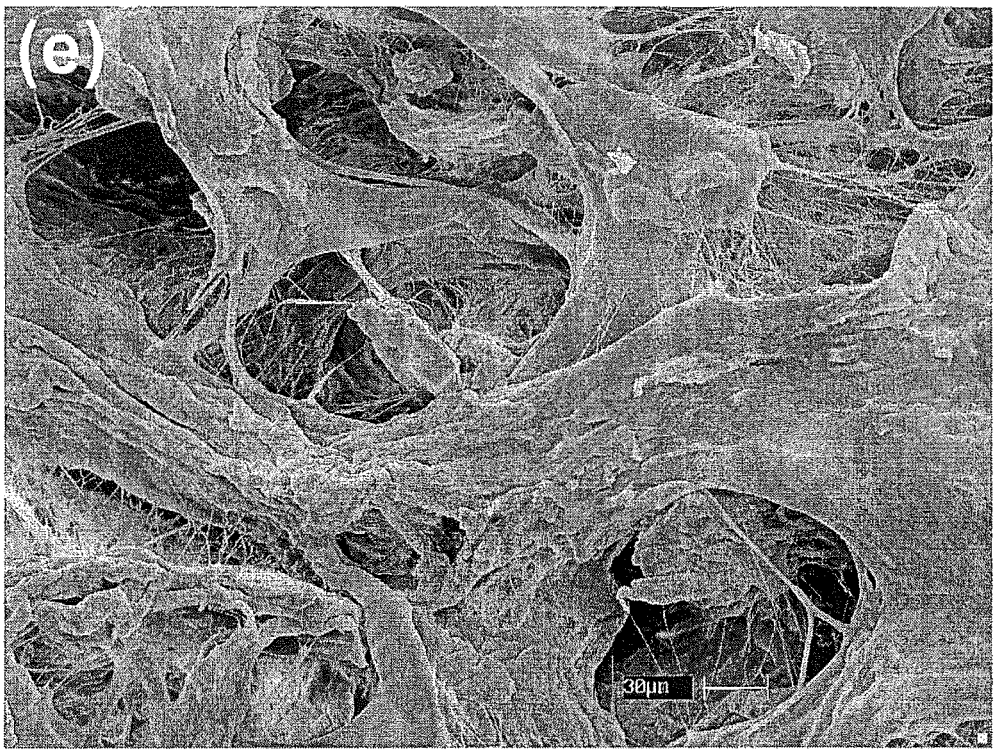
(b) Acetone washed ground leather shavings



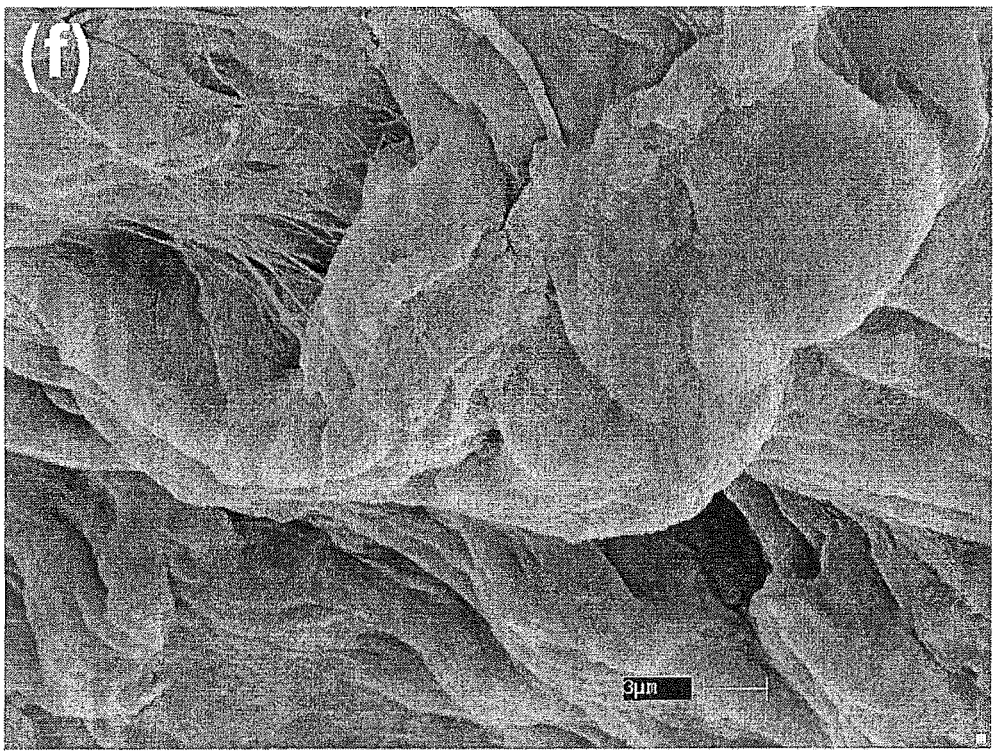
(c) Autoclaved leather shavings



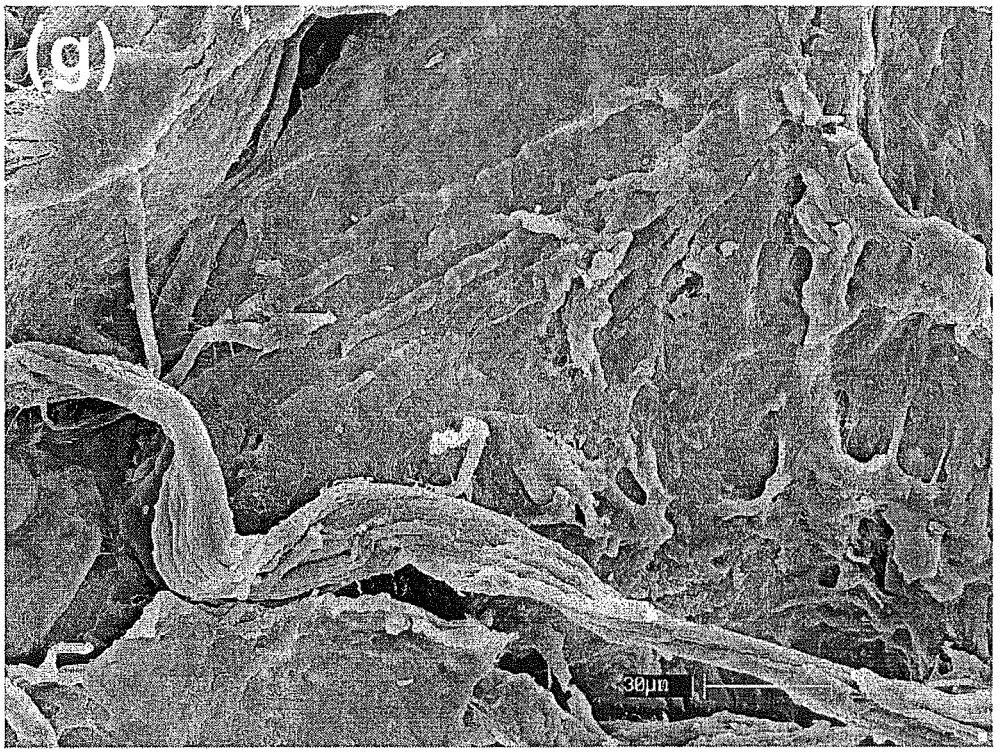
(d) Autoclaved leather shavings



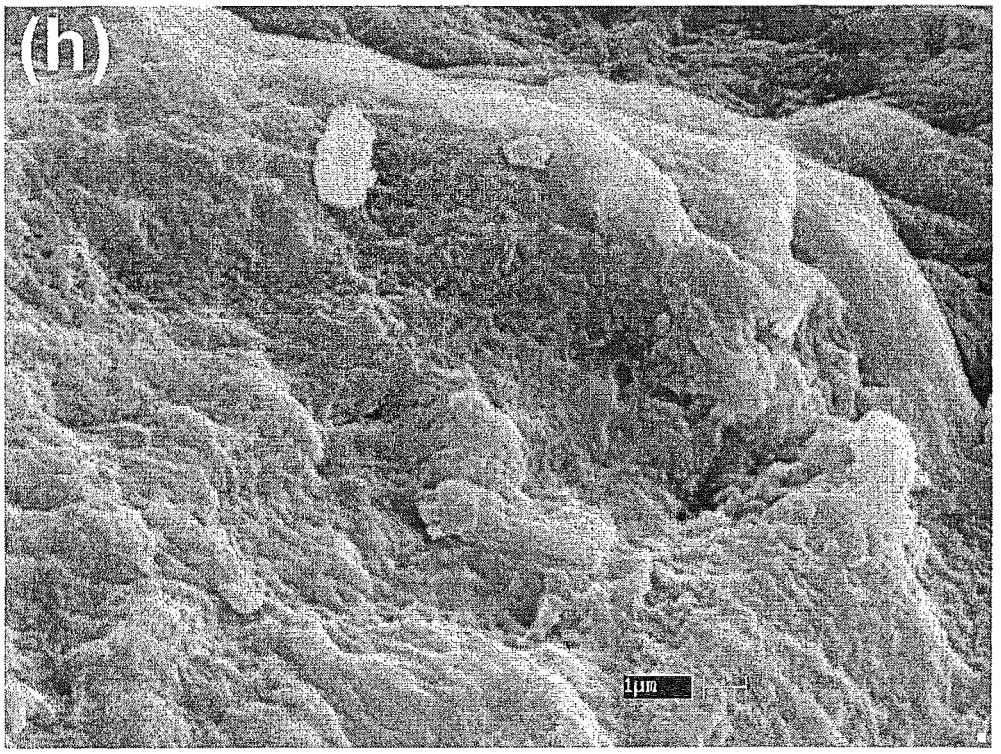
(e) 25mM HCl washed leather shavings



(f) 25mM HCl washed leather shavings



(g) Hide powder



(h) Hide powder

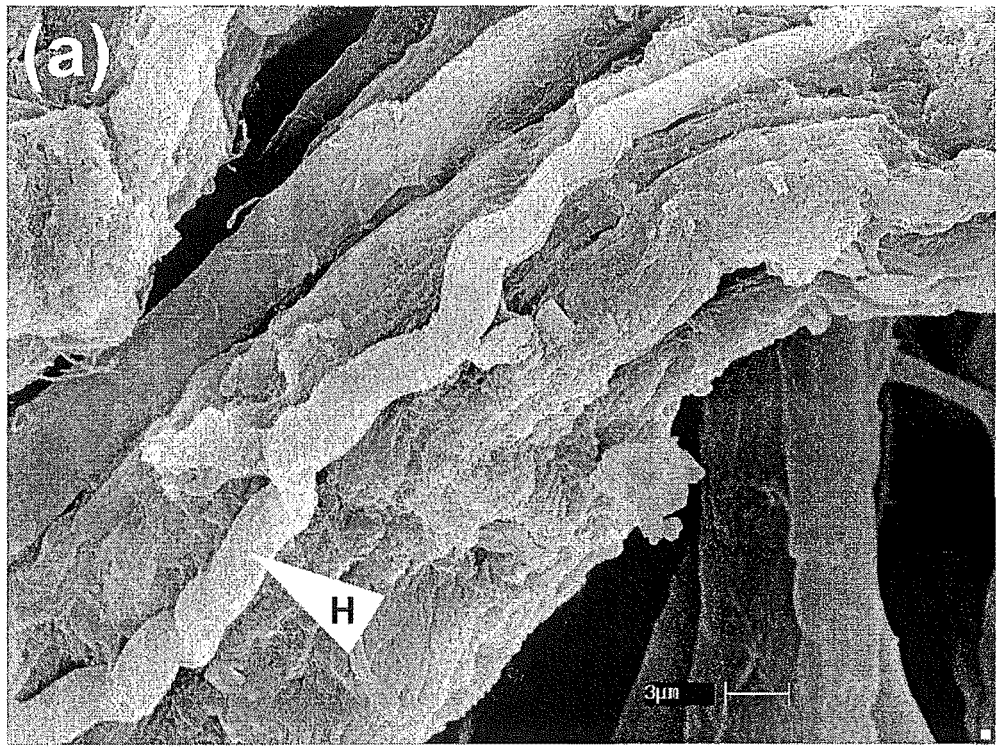
3.5.2. Structural changes as effected by microbial decomposition

Extensive microbial growth was observed on leather substrates decomposed for 9 months (Figure 3.14). Fungal hyphae (H) and possibly actinomycetes (1-2 μm diameter, A) were seen on most substrates (Figure 3.14 (a) & (c)). Fungal hyphae and bacteria had collapsed in air-dried samples, but were visible on critical-point dried or frozen samples. Cocci bacteria (B) were only observed on leather decomposed in the soil medium, as shown in Figure 3.14 (f).

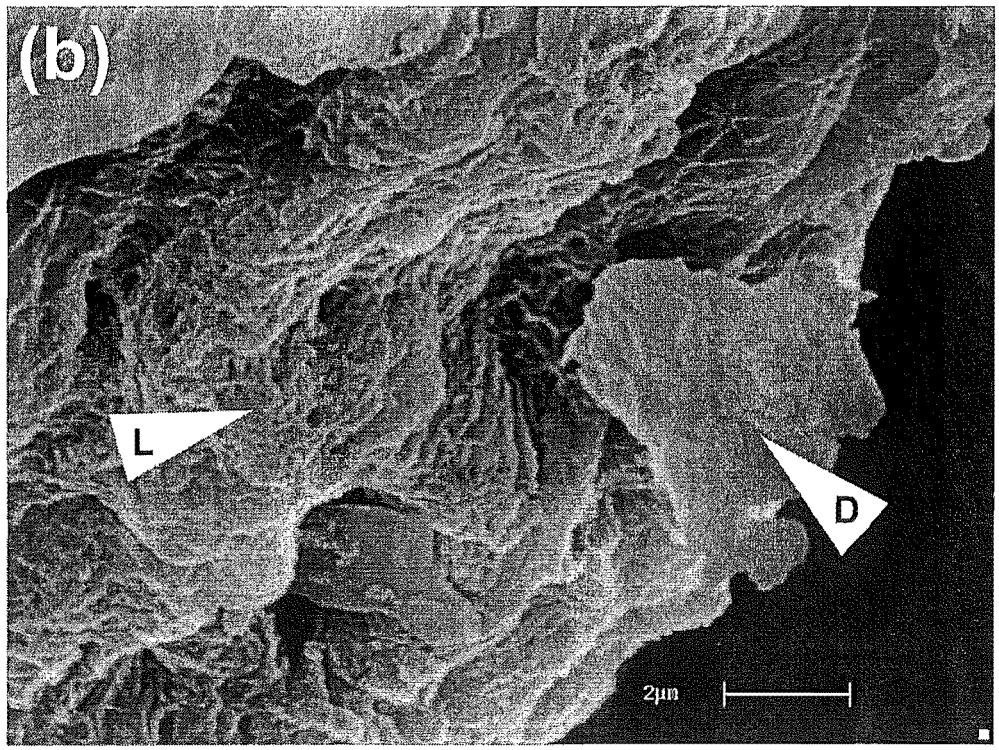
The treatment of each substrate appeared to have minimal effect on the extent of microbial growth visible. Unwashed and acetone washed ground shavings exhibited similar structural degradation when examined at high magnification (Figure 3.14 (b) & (e)). The highly ordered fibril structure of the leather was degraded and the surface deeply pitted. Individual collagen fibres were unravelled from bundles and could be seen clearly (Figure 3.14 (i)). Surface debris, possibly clay particles (C) or partially degraded collagen (D) was observed on acetone washed samples and on unwashed samples in a soil medium (Figure 3.14 (d) and (f)).

Fungal reproductive structures were observed on unwashed samples supplemented with an additional carbon source, glucose (Figure 3.14 (g) and (h)). These have been tentatively identified as derived from *Aspergillus* and *Trichoderma* respectively. Microbial growth obscured the underlying leather structure. Due to the poor quality of negatives for these two samples, digital enhancement has been used.

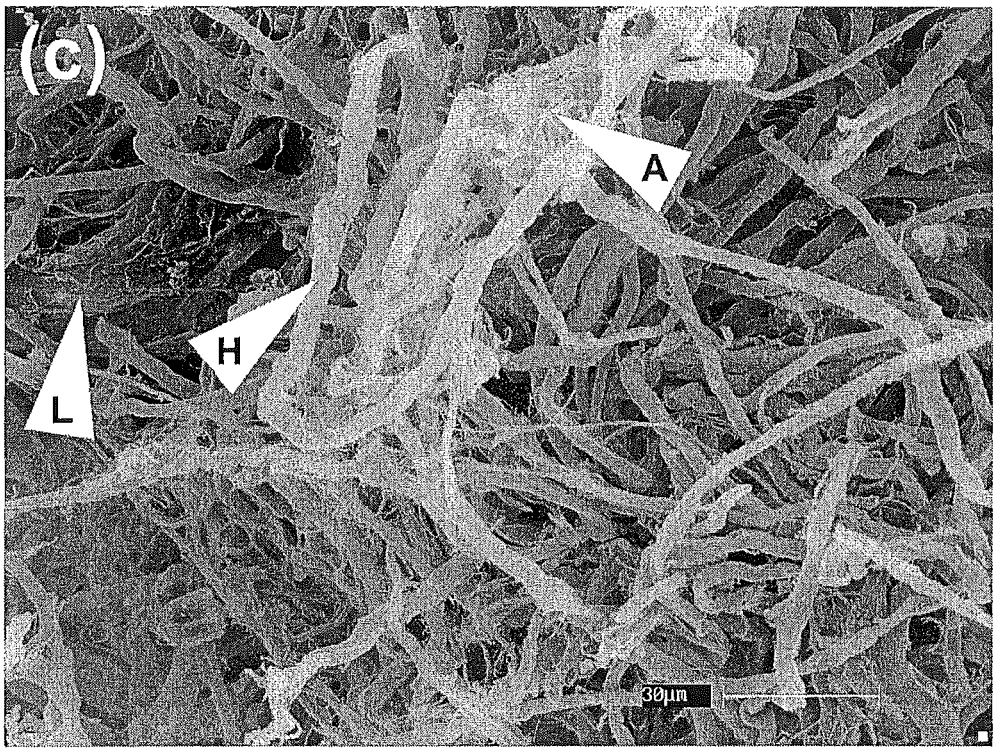
Figure 3.14 : Scanning electron micrographs of decomposed leather (9 months) showing substrate degradation and microbial growth.



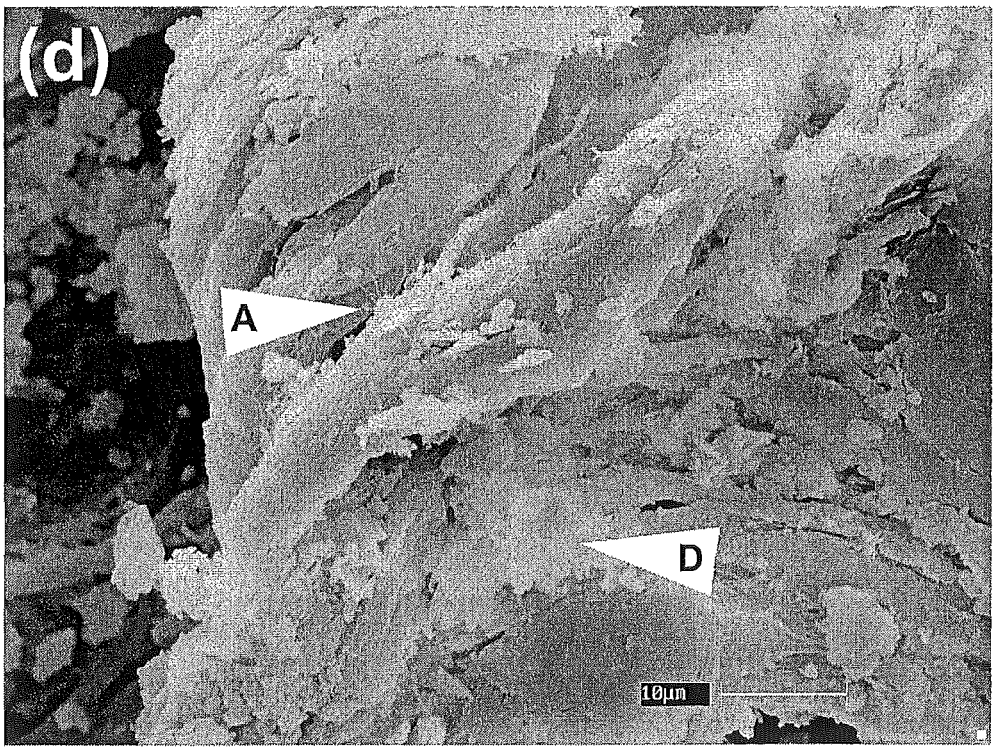
(a) unwashed ground leather shavings; H – fungal hypha



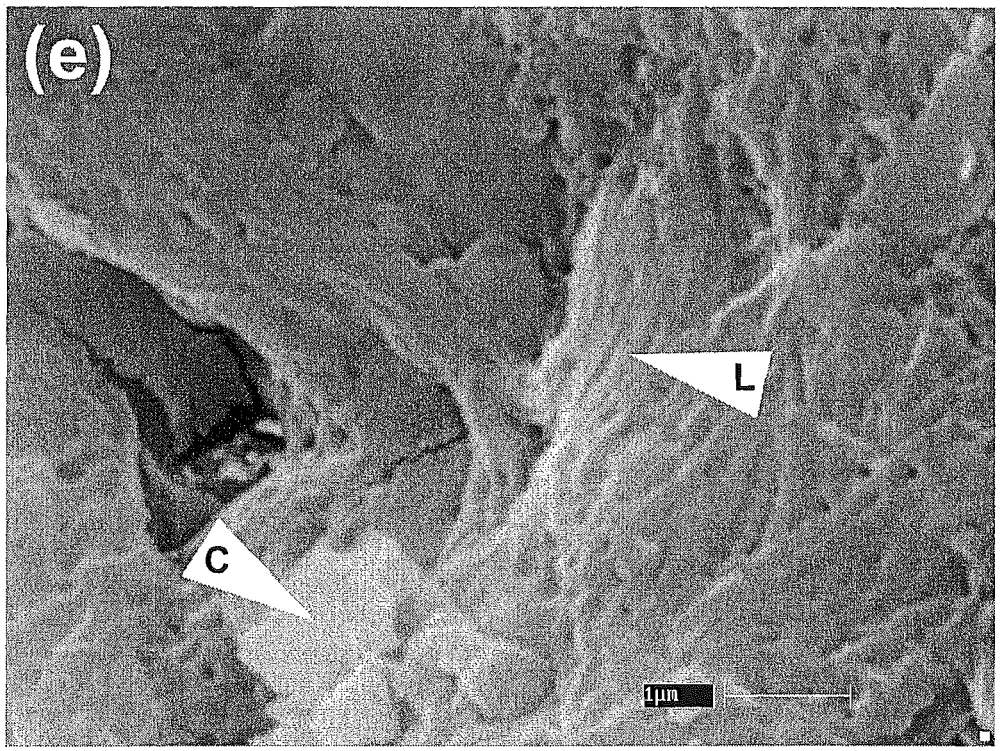
(b) unwashed ground leather shavings; D – Debris, possibly collagen
L – Leather substrate



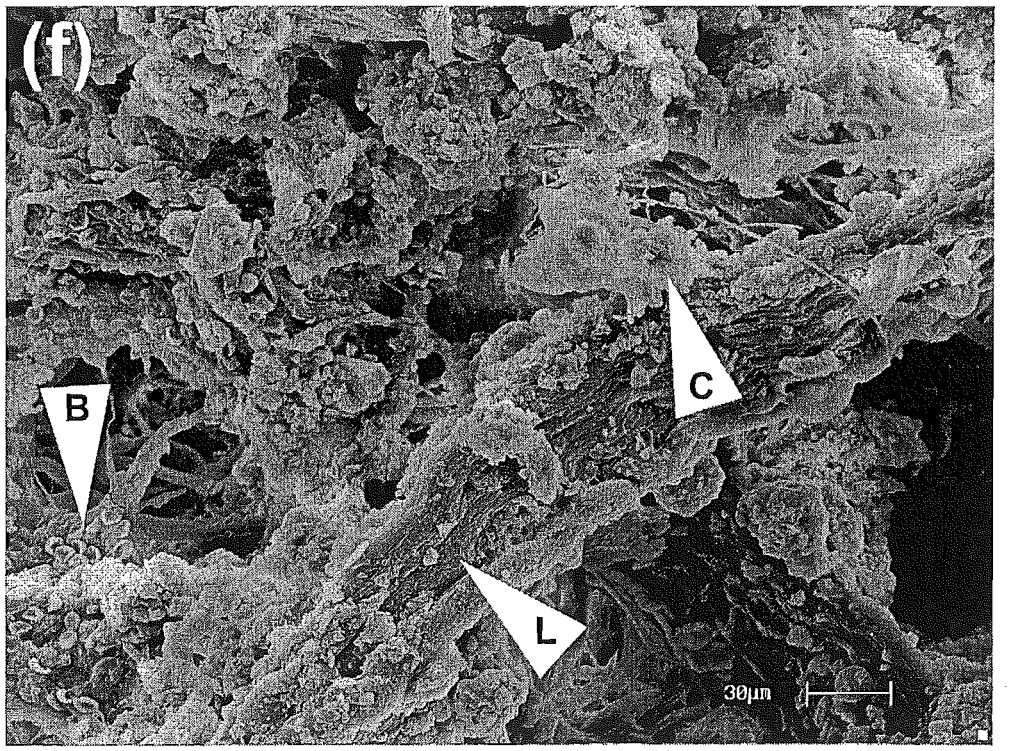
(c) water washed ground leather shavings; A – Actinomycetes H – fungal hyphae; L – Leather



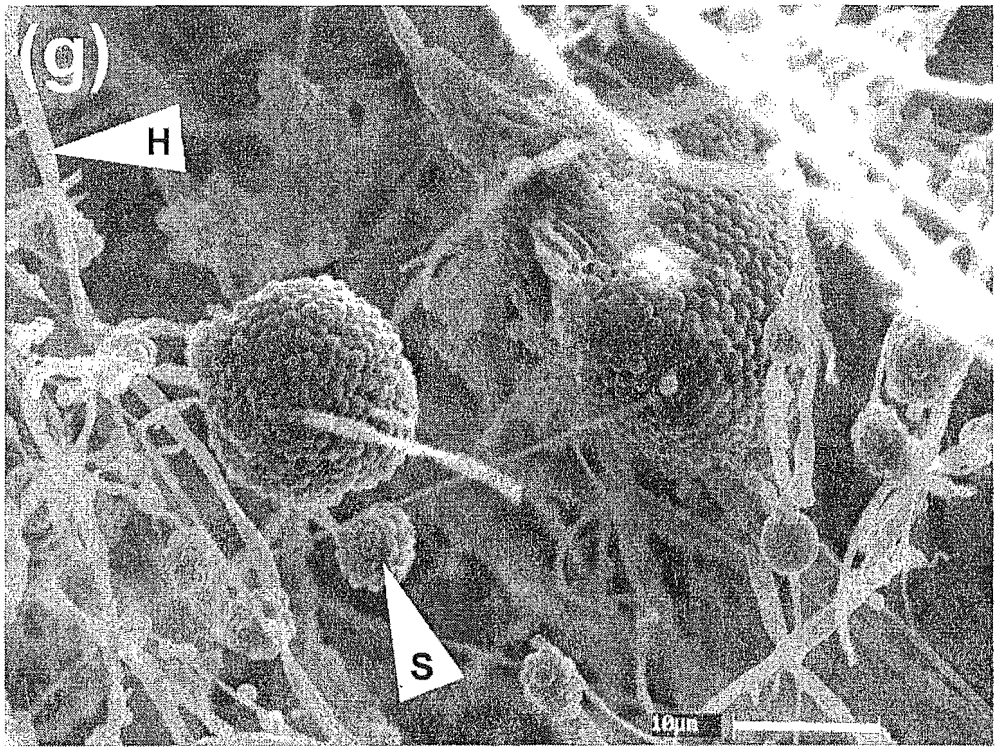
(d) acetone washed ground leather shavings; A – Actinomycetes D – Collagen debris



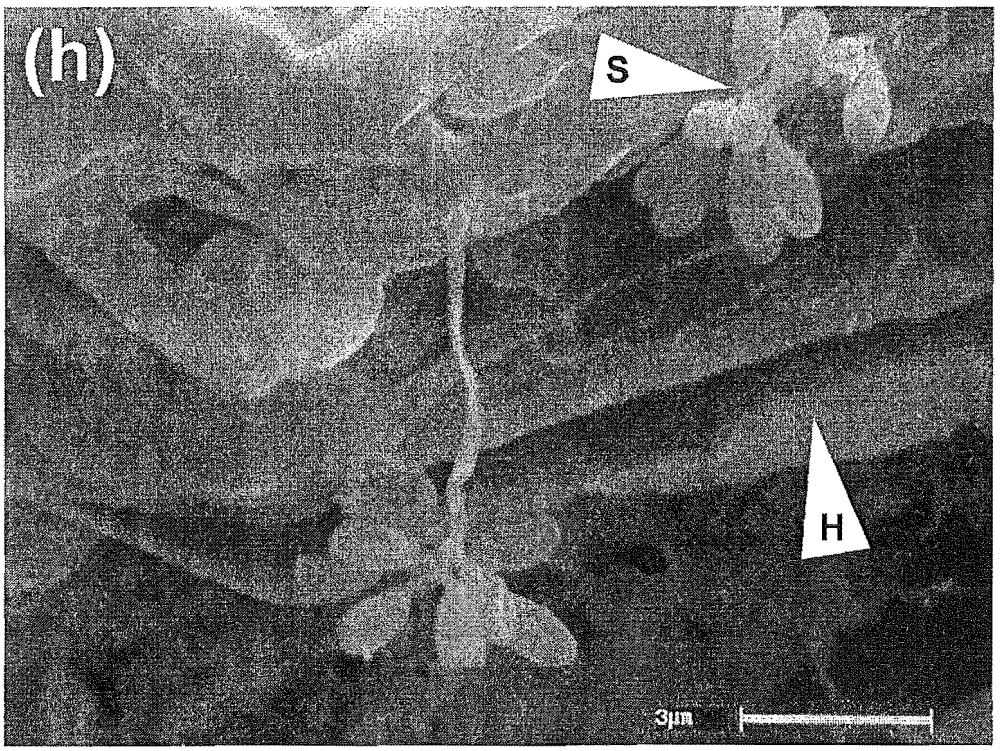
(e) acetone washed ground leather shavings; C – Clay particles L – Leather



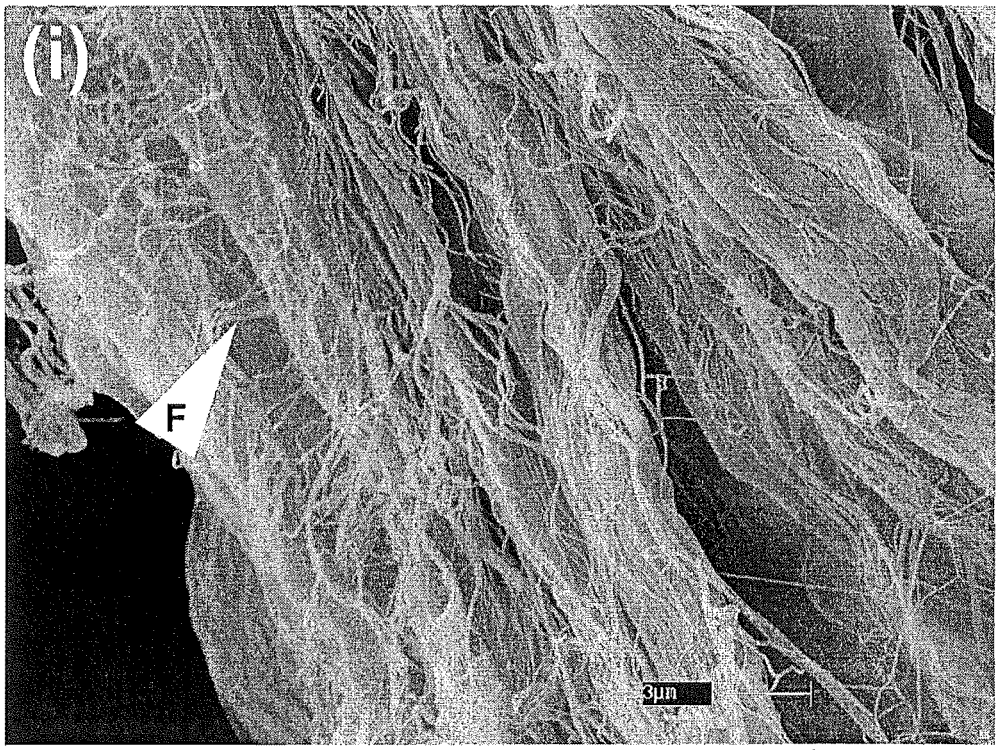
(f) unwashed ground leather shavings in Cass soil; B – Bacteria; C – Clay particles; L – Leather



(g) unwashed ground leather shavings with glucose
H – fungal hyphae; S – Fungal sporing structures



(h) unwashed ground leather shavings with glucose
S – Fungal sporing structures



(i) unwashed ground leather; F – Collagen fibrils

3.6 Plant seedling germination in the presence of chromium salts

Cucumber seeds were able to germinate, to varying degrees in the presence of both Cr(III) and Cr(VI) salts, with the mean radicle length obtained shown in Table 3.14. Solution pH was less than 4 for both Cr(III) and Cr(VI) salts, with chromic oxide (Cr (VI)) at 1.8.

Cr(VI) salts affected seed germination greatly with radicle length averaging 2.6 mm for chromic oxide, and 8.8 mm for potassium dichromate. A slight browning and thickening of radicles and incomplete leaf emergence was observed. Lateral branching of root system was also limited.

Cr(III) salts affected seed germination to a lesser degree. Chromium nitrate was the most damaging, with a mean radicle length of 34.2 mm. Minimal inhibitory effect was observed for chromium sulphate (58.8 mm), although germination strike rate was lower (89 %).

All Cr(III) germinated seeds exhibited normal plumule and radicle development, and radicles were laterally branched with fine root hairs evident.

Seedling radicles can be seen in Figure 3.15.

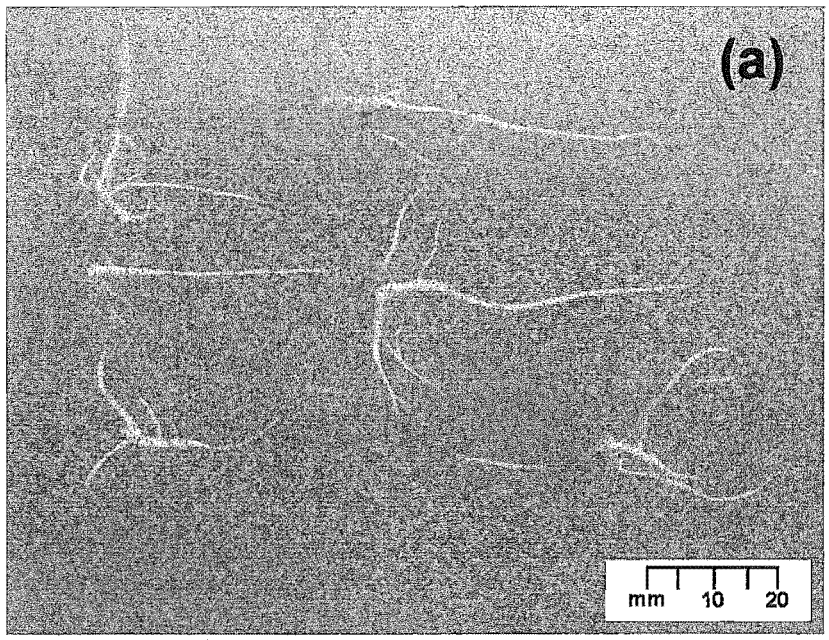
Table 3.14 : Germination of cucumber seeds in presence of 1000 ppm soluble Cr, as observed by radicle length.

Chromium solution (1000 ppm)	Chromium oxidation-state	Solution pH	Mean radicle length (mm)	Seed germination strike rate (%)
Chromium nitrate	III	3.1	34.2	100
Chromium chloride	III	3.6	42.8	100
Chromium sulphate	III	3.1	58.8	89
Potassium dichromate	VI	3.9	8.8	100
Chromic oxide	VI	1.8	2.6	100
Distilled water	-	6.6	54.0	100

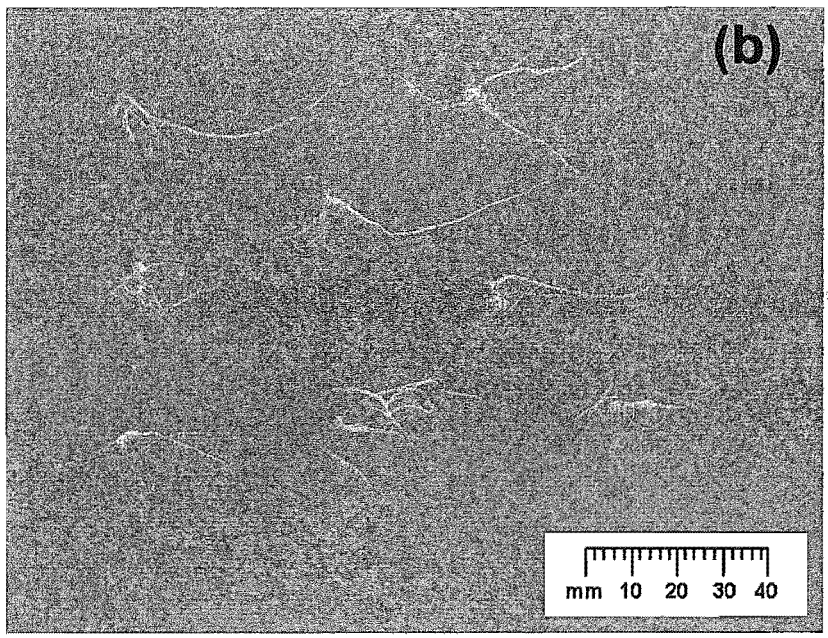
Note

- Standard error of radicle length did not exceed 11 mm.

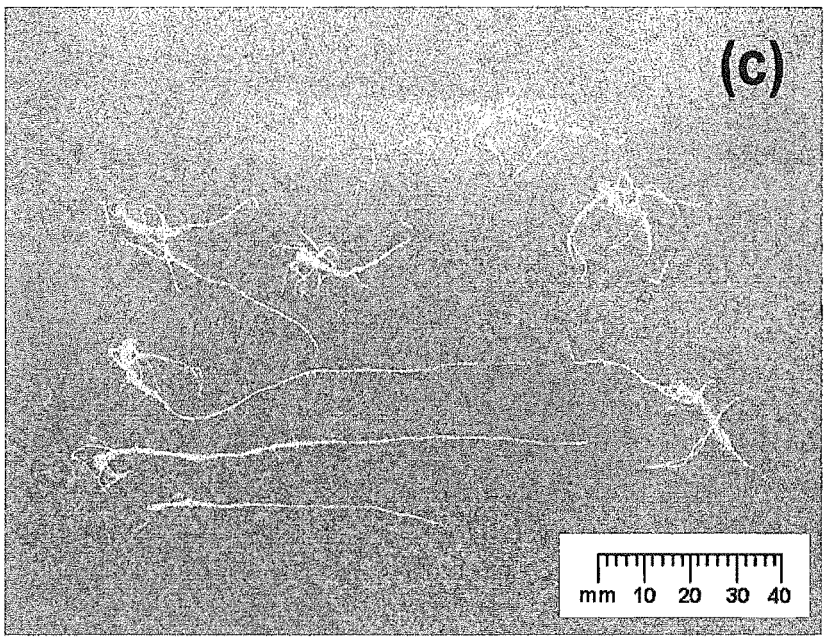
Figure 3.15 : Cucumber seeds germinated in the presence of 1000 ppm Cr(III) / Cr(VI).



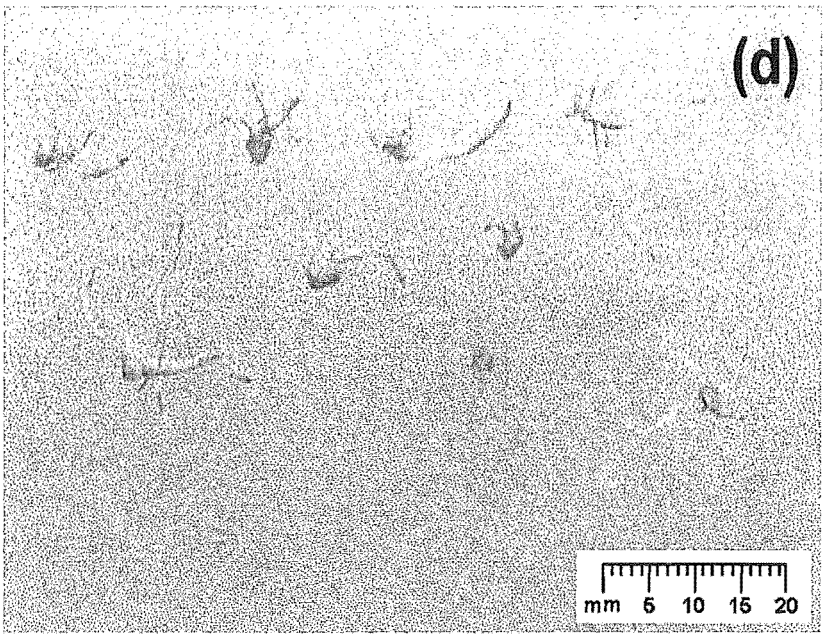
(a) chromium nitrate – $Cr(NO_3)_3.9H_2O$



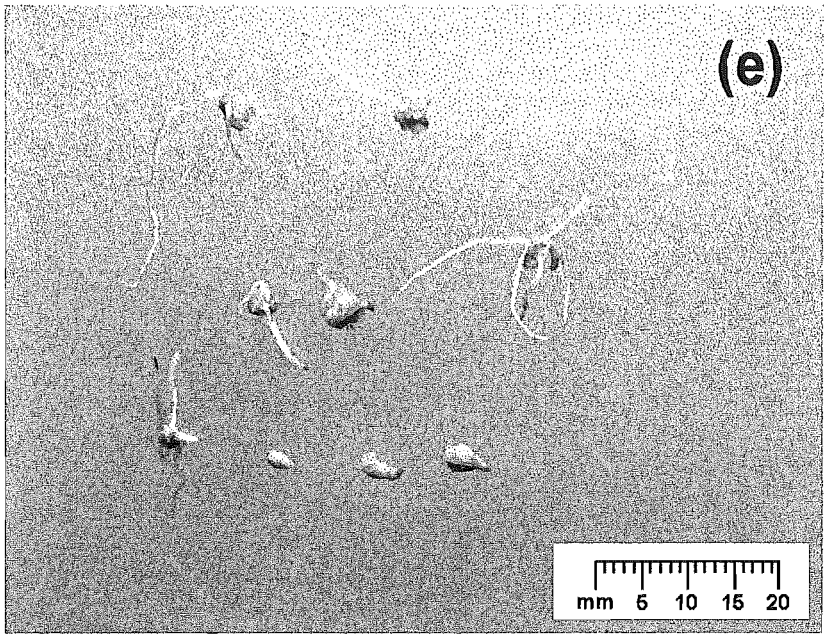
(b) chromium chloride – $CrCl_3.6H_2O$



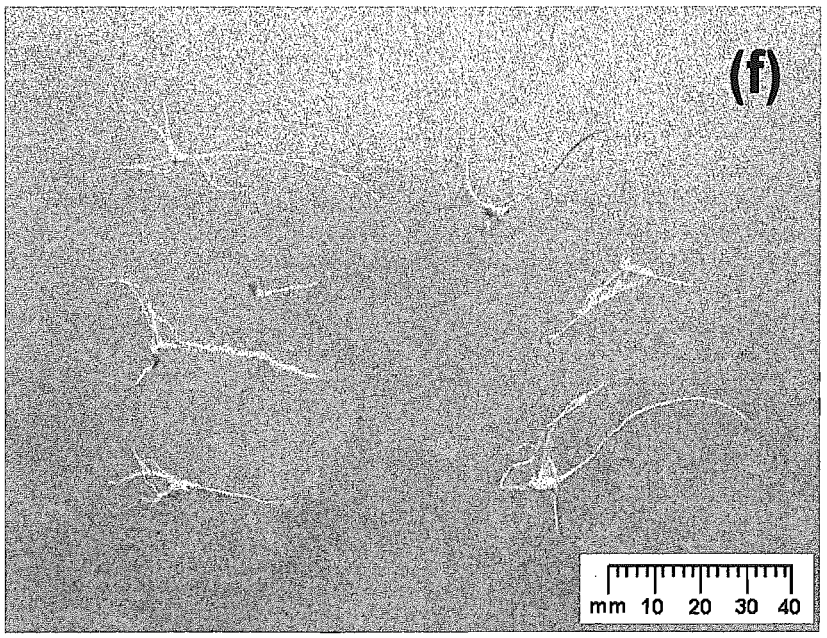
(c) chromium sulphate – $Cr_2(SO_4)_3.xH_2O$



(d) potassium dichromate – $K_2Cr_2O_7$



(e) chromium trioxide – CrO_3



(f) distilled water

CHAPTER 4 : DISCUSSION

The manufacture of leather produces an immense quantity of waste, with only 200 kg of leather produced for every tonne of raw hide (Taylor *et al.*, 1997). Currently, the majority of this waste (500,000 tonnes worldwide per annum) is disposed of in landfill. In New Zealand, the amount of waste is unknown, as most of the leather leaves the country in the 'wet blue' state for processing overseas. However, the waste that is generated here is disposed of in landfill (DasGupta, 1996). The waste is particularly high in nitrogen (*ca.* 16 %) and attempts have been made to utilize this.

In this thesis, the microbial and enzymatic degradation of leather waste was studied with the aim of determining if and how leather could be decomposed, and whether it might be a useful fertilizer supplement.
An extensive literature search revealed little information on the decomposition of leather by mixed microbial populations. Most of this work has been undertaken by Indian and Chinese scientists, and their reports are often difficult to follow or inadequately describe the experimental work, allowing no real conclusions.
Models for the decomposition of animal matter would have been more useful but only very few studies have been reported (Waksman, 1952).

4.1 Microbial decomposition

Normally, substrates with a low C:N ratio (C:N ratio of leather is 2.0) would be predicted to be of excellent resource quality and easily degraded. However, the tanning treatment, whereby chromium is covalently bound within the collagen matrix making up the leather, effectively lowers the quality of the substrate, limiting microbial degradation. The nature of the chromium-leather complex is still not precisely known (Gayatri *et al.*, 1997), although more

scientific information is emerging, as the manufacture of leather moves from an art to a science (Brown *et al.*, 1997a).

In plant litter systems, decomposition releases mineral nutrients into the soil. This leads to microbial growth and the formation of soil organic matter (Swift *et al.*, 1979). Usually decomposition of plant litters is limited by the availability of nitrogen as substrates generally have high C:N ratios of 30 to 50 %.
This inherent resource quality and the nature of the physical / chemical environment in which the substrate is found may regulate the degree of decomposition (Swift *et al.*, 1979).

During decomposition, substrates are degraded by microorganisms, with the mineralization of essential elements and the formation of soil organic matter (Swift *et al.*, 1979). Water soluble nitrogenous fractions of the substrate are generally the first to be metabolised. An increase in microbial biomass occurs as zymogenous organisms rapidly increase in number and metabolise the available carbon within the water soluble fraction. Carbon is lost continuously through mineralization to CO_2, as the microbial biomass degrades the substrate, lowering the substrate C:N ratio.
Net mineralization of nitrogen occurs when the quantity of mineralized-N exceeds the microbial decomposer population's nitrogen requirement. This may occur in the later stages of decomposition as autochthonous soil microorganisms assimilate and catabolize the existing zymogenous population and the more recalcitrant fractions of the substrate (Waksman, 1952; Dickinson, 1974; Williams and Gray, 1974; Swift *et al.*, 1979).
During research for this thesis, decomposition experiments were performed using *in vitro* microcosms to simulate individual environmental factors and their effect on the decomposition process, as complex conditions found *in situ* make it difficult to determine the relative importance of each factor (Nykvist, 1961).

In vitro experiments allow for variability in one environmental factor while holding all others constant. By performing a series of experiments examining individual factors and extrapolating results to the *in situ* situation, a decomposition model may be constructed.
This is often difficult to do reliably, as environmental factors may interact, obfuscating their importance to the model. Misrepresentation of the *in situ* decomposition process may also result from an inability to accurately assess the importance and / or presence of individual environmental factors.

In this study, changes to resource quality were made by washing or grinding samples, and the physio-chemical effects of temperature, moisture, soil type, and aeration were examined. The rate of decomposition is related to the environment in which the substrate is found, with temperature often a major controlling factor (Williams and Gray, 1974). Seasonal changes in temperature have been linked with changes in microbial activity, with maximal activity reported in warm, moist conditions (Clarholm and Rosswall, 1980).

Although temperatire is closely related to moisture content under natural conditions, by strict control of moisture levels, the effect of temperature could be observed. Results from both short (30 day) and long term (330 day) decomposition experiments examining the effect of temperature on decomposition suggested that decomposition occurred more readily at temperatures in excess of 20 °C. (see Figure 3.5 and Table 3.3). This is consistent with the view that the majority of soil microorganisms are mesophilic and show increased enzyme activity, all things being equal, with increased temperature as described by the Arrhénius equation (Dickinson, 1974). Consistent with observations by Clarholm and Rosswall (1980), less nitrogen was mineralized at low temperature (less than 10 °C) than at the mesophilic 20 °C.
Long term leather containing microcosms incubated at variable temperatures, were found to mineralize more nitrogen than those incubated at constant

temperature (see section 3.3.1). Upon reincubation at elevated temperatures following periods of cooling, microbial succession utilizing the release of immobilized nitrogen within the biomass is likely to have occurred, resulting in an increase of mineral-N (Nicolardot *et al.*, 1994).

In this study, the availability of moisture was found to affect decomposition processes, with minimal decomposition occurring at low moisture levels. Moisture is a requirement for microbial growth, although high levels may prevent gas diffusion within the soil, promoting anaerobic conditions. Low moisture can reduce net nitrogen mineralization by restricting the diffusion of metabolites away from the site of decomposition (Richards, 1987). This study showed that the requirement for moisture was secondary to temperature (Figure 3.5).

Results from the decomposition of washed leather samples showed that the acetone washing increased the susceptibility of the substrate to microbial degradation. This may have been due to an opening of the leather matrix and the partial solubilization and removal of pesticides, with both Preventol WB and TCMTB found in solvent extracts[1]. Comparison of electron micrographs between unwashed and acetone washed shavings (Figure 3.13) confirmed that the acetone caused denaturation of the leather structure, with an unravelling of collagen fibrils seen in washed samples (b), and intact leather in unwashed samples (a).

Water-washed ground leather was found to mineralize nitrogen similarly to unwashed leather. This may reflect the higher pesticide content of water-washed leather samples compared to acetone washed samples. The pesticides form emulsion in water, but after incorporation into the leather during the 'pickling' stage of tanning, they may become difficult to remove with the action of water (Muthusubramanian *et al.*, 1998).

[1] Courtesy of Chemistry Department, University of Canterbury

For unwashed ground leather samples decomposed in sand and soil matrices, results indicate that both Cass and Ilam soils contain sufficient quantities of available carbon and nitrogen (Ilam, 5.8 %C, 0.58 %N; Cass, 0.23 %C, 0.19 %N) to allow degradation of the leather substrate. In these experiments, it was assumed that the mineralization of the soil was organic matter was similar in control and experimental flasks.

The decline in mineral-N seen at day 90 (Figure 3.3) was thought to represent a period where the moisture levels in microcosms were low. Adjustment to 30 % moisture and reincubation resulted in an increase of mineral-N at day 180, at which time 3.8 % of the total nitrogen had been mineralized in the case of acetone washed leather.

In waterlogged soil conditions where gas diffusion is limited, anaerobic conditions may exist (Richards, 1987). Under anaerobic conditions, the efficiency of substrate utilization by the microbial decomposer population is reduced (de Laune *et al.*, 1981), with either fermentation of the substrate, releasing methane and hydrogen, or denitrification of previously formed nitrate.

Under conditions of anaerobiosis, acetone washed leather mineralized less nitrogen than under aerobic conditions (*ca.* 2 %). Results from anaerobically incubated microcosms (less than 0.5 % N-mineralization), suggest that denitrification of mineral-N had occurred. This is consistent with bacterial denitrification of nitrate to gaseous nitrous oxide or molecular nitrogen, resulting in a net gain of metabolic energy (Richards, 1987).

The addition of available carbon (glucose) or nitrogen (NH_4NO_3) to microcosms containing leather did not increases levels of nitrogen mineralization, although extensive microbial growth was observed in glucose amended microcosms (Figure 3.9). This observation suggests that available nitrogen in glucose-amended flasks was limited, resulting in the

immobilization of nitrogen as biomass-N and it's tight recycling (Jones and Richards, 1978).

No visible microbial growth or mineralization of nitrogen occurred in nitrogen amended microcosms, suggesting a limitation of available carbon in the system. This is contrary to previous reports, in which the addition of ^{15}N-labelled plant residues to soil has been shown to result in increased microbial growth and nitrogen mineralization (Paranas, 1975), suggesting that the substrate quality of leather is quite unlike that of plant litter.

Taken together with the result from carbon-amended microcosms, it is suggested that leather is of poor resource quality, with only minimal levels of available carbon and nitrogen, and that these levels sustain only a small and not very diverse biomass. Several colonies of bacterial Gram positive cocci and Gram negative rods were isolated, and fungi were tentatively identified as *Trichoderma* and *Aspergillus* spp. Results from weight loss studies in which leather was supplemented with carbon (glucose), support the finding that decomposition does occur when there is readily available carbon, with 12-14 % weight loss in 30 days for carbon amended samples. The observed N-mineralization differences between leather samples and autoclaved leather may result from alteration to the physical structure of the leather as well as limited chemical hydrolysis by water and acetone washing (Figure 3.13).

Steam hydrolysis of collagen has previously been shown to result in the formation of gelatin (Gustavson, 1949), a manufactured protein that has a similar chemical composition to collagen (Appendix D) but is more amenable to proteolytic enzymes (Seifter and Harper, 1970). Gelatin has been reported to degrade slowly with purified collagenases, and that the tertiary structure of collagen at the cleavage site is very important to facilitate the action of collagenase (Cawston and Murphy, 1981). Therefore, microbially derived enzymatic action on autoclaved leather or gelatin is more likely to involve nonspecific proteases rather than collagenases.

In this study, it was found that prior to 20 days incubation, very little nitrogen in autoclaved leather was mineralized. This may be explained by microbial

populations using this time to adapt to nonfavourable environmental conditions, with selection pressure enriching for those organisms capable of growing in the presence of chromium

Nitrogen mineralization after 20 days was thought to have resulted from decomposition of the autoclaved leather, once the tolerant microbial population was of sufficient size to degrade the leather. This is consistent with the findings of (Frostegård *et al.*, 1996), in which sensitive organisms were eliminated on initial incubation of microcosms containing heavy metals. It is expected that the treatment of leather by autoclaving (121 °C, 20 mins) will have significantly decreased the antimicrobial activity of the fungicides found in the leather, with thermal decomposition (hydrolysis) of the active compounds occurring at temperatures in excess of 80 °C (Halligan, 1998).

The persistence of antimicrobial activity by the fungicides Preventol WB® and Busan 30LW®, was observed during the decomposition of casein by soil microorganisms (see Figure 3.6). Significant inhibition of casein N-mineralization occurred at fungicide levels that were two orders of magnitude less than recommended application rates of 0.1 % w/w. Using casein as a model for leather decomposition, it is suggested that incomplete removal of antimicrobial compounds (such as TCMTB and phenolics) had affected the decomposition of leather in all decomposition experiments.

Further evidence for this conclusion was gained using chromium and pesticide free hide powder, which was extensively decomposed after 30 days incubation (53 % of total nitrogen mineralized). Greater than 50 % mineralization over a short period of time is consistent with models for the decomposition of high quality substrates, such as animal matter (Waksman, 1952), which is what hide powder is.

The presence of unwashed ground leather had no significant effect on the extent of nitrogen mineralization of added substrates (see Figure 3.8). Autoclaved leather, which has a high content of soluble-N (85 % soluble in buffers), was found to increase overall levels of decomposition in the 30 day

study (see Figure 3.7). High levels of nitrogen mineralization in microcosms containing autoclaved leather were due the increased substrate quality compared to unwashed leather. The high levels of available nitrogen and low C:N ratio are probably responsible for this (Table 3.1).

Net mineralization was not observed for *Quercus ilex* and *Liriodendron tulipifera* flower litter, although the observation of extensive microbial growth suggests that any mineral-N produced was immobilized in microbial tissue. Both are relatively poor substrates compared to the autoclaved leather and have high C:N ratios (25-30). Observations of growth, are consistent with the finding that in concomitant decomposition, decomposition of both substrates will occur, but nitrogen will be immobilized and tightly recycled when the two substrates are of significantly different resource quality (Swift *et al.*, 1979).

The ability for microbial decomposition of substrates to occur in the presence of leather, suggests that general decomposition processes are not unduly affected by leather and it's constituents. This may reflect the low water soluble fraction of the leather (8.3 % with hot water extraction), resulting in the leather substrate behaving as an inert matrix. Water soluble fractions were found to contain minimal nitrogen (less than 0.7 % total fraction N), with the bulk of the extract believed to contain surfactants and soluble carbonaceous compounds. Microbial growth in the presence of WSS was observed, suggesting that compounds leachable with water are not inhibitory to microbial growth.

Microbial growth was often difficult to see in decomposition studies, but analysis with electron microscopy (Figure 3.14) showed the presence of fungal hyphae and actinomycetes in many microcosms. Bacteria were not obvious in the majority of sand microcosms examined with SEM, and it is thought that the proliferation of hyphae may have obscured the bacteria, or that bacterial cell walls had collapsed.

4.2. Enzymatic decomposition

There are numerous reports in leather industry trade journals regarding the enzymatic hydrolysis of chromium tanned leather. The majority of these papers focused on the use of alkaline proteases, with the production of protein hydrolysates and the recovery of chromium. Furthermore, the treatment of leather with hot alkali (60-70 °C, pH 8.3-9.0) has previously been reported as a prerequisite for enzymatic action (Taylor *et al.*, 1992; Brown *et al.*, 1996; Cabeza *et al.*, 1997; Chakraborty and Sarkar, 1998). For example, Brown *et al.*, (1996) advocated the use of alkaline proteases (pH 8.3-10.5, 60 °C), as it was found that Cr(III) was insoluble and precipitated as $Cr(OH)_3$, allowing for the formation of chromium free protein hydrolysates and an almost complete recovery of the chromium.

In this study, several experiments were used to determine the action of the extracellular enzymes collagenase and pepsin on ground leather and on the 2 mm^3 materials. Individually, the enzyme action was similar for both enzymes (see Figure 3.10(a)). However, both leather samples were found to be readily degraded by the combined action of collagenase and pepsin, without the need for pretreatment. When collagenase preceded pepsin, in addition to the leather samples, more than 95 % of the total-N in ground leather samples was hydrolysed, and 50 % for 2 mm^3 pieces.

The combined action of pepsin preceding collagenase, was found to be significantly less, with 30 % and 5 % dissolution of ground and 2 mm^3 leather samples respectively. Together with the low levels of enzymatic degradation in the single enzyme study (less than 10 %), it is suggested that pepsin is unable to degrade the collagen within the leather substrates without initial collagenolytic action. The results, in Figure 3.10(b), also suggest that the proteolytic activity of collagenase is highly specific and limited in nature. This is consistent with the known activities of these two enzymes (Seifter and Gallop, 1962; Seifter and Harper, 1970; Cawston and Murphy, 1981; Fersht, 1985).

Collagenase is highly specific for the Gly-Leu and Gly-Ile bond approximately three quarters of the way along from the N-terminal region of the collagen molecule. Depending on the source of the enzyme, either one or all three α-chains in the collagen helix will be hydrolysed at this bond. Microbially derived collagenases have been reported to hydrolyse the collagen into both high and low molecular weight peptides (Seifter and Harper, 1970). This endo-proteolytic activity is in contrast to the exo-proteolytic activity of pepsin. The activity of pepsin and other common proteolytic enzymes towards native collagen is thought to only occur at the globular N-terminal region of the molecule, leaving the rest of the helical molecule untouched (Seifter and Harper, 1970). Therefore, the extensive release of leather-N by the cumulative action of collagenase and pepsin can be explained by the proteolytic action of pepsin on peptide fragments generated by collagenase.

The pretreatment of the leather samples by washing with water or acetone, or grinding of the shavings, appeared to be unnecessary, since there was no significant effect on dissolution of the leather samples with respect to method of sample preparation. However, the small pieces (2 mm^3) of leather were not completely solubilized, with 50 % of their nitrogen hydrolysed. In contrast, autoclaved leather samples were extensively hydrolysed by both the buffer system and enzymes. The highly stable triple-stranded coil structure of the collagen in the leather is thought to have been denatured by the action of steam hydrolysis (autoclaving). The thermal decomposition of leather would involve the breaking of covalent cross-links and hydrogen bonds in the collagen, allowing solubilization of the protein. Insignificant solubilization (*ca.* 8 %) of hide powder controls was observed for phosphate buffer (pH 7.4), but after the addition of 25 mM HCl, solubilization had increased (to 24 %). This is consistent with evidence using electron micrograph analysis of acid washed leather (Figure 3.13(e) & (f)), which showed extensive denaturation of the leather.

The observed increase in dissolution of denatured leather samples by buffers may have resulted from an overestimation of leather solubilization. This was

most likely caused by experimental error in precipitating soluble proteins at their isoelectric point. An isoelectric point of 4.5-5.0 was assumed for all samples, including hide powder and autoclaved leather (Gustavson, 1949). Commercial hide powder, as used in this study, is made from the limed skins prior to chrome tanning, whereas native unlimed collagen has an isoelectric point of 7.0-7.5. The difference of 2 pH units in the isoelectric point between native hide powder and tanned leather is due to the action of alkali on collagen, releasing carboxyl groups during the process of 'liming' (Gustavson, 1949).

Poisoning of the enzymes in leather hydrolysis experiments was not observed, in agreement with the presumed unavailability of the Cr(III) ion (Nieboer and Jusys, 1988). Results from hide powder studies examining the effect of chromium oxidation state on the enzymatic action of pepsin and collagenase, suggest that freely available Cr(III) at neutral pH is inhibitory to collagenase (see Figure 3.12). In phosphate buffer (pH 7.4), available Cr(III) was observed to inhibit collagenase activity towards hide powder at concentrations as low as 10 ppm. Chromium would have existed as $Cr(OH)_2^+$, and may have formed polymers with linking of chromium ions via hydroxyl bridges (Nieboer and Jusys, 1988). Increases in the size of chromium complexes may have inhibited enzyme action through steric interference at the collagenase active site. In contrast, commercially tanned leather contains *ca.* 40,000 ppm Cr(III) and this did not appear to inhibit collagenase activity. This suggests that when chromium is tightly bound within the leather matrix, it does not interfere with the activity of collagenase, and remains bound to the peptide fragments produced. However, when Cr(III) is freely available in solution, poisoning of collagenase activity ensued.

Cleavage of the collagen molecule by collagenase may have left chromium bound to collagen, allowing for extensive hydrolysis by pepsin without

inhibition by $Cr(H_2O)^{3+}$, the likely form of chromium at pH 2 (Nieboer and Jusys, 1988).

No inhibition was observed for Cr(III) towards pepsin (pH 2). The hydrated ion of Cr – $Cr(H_2O)^{3+}$ is nonlabile with respect to ligand exchange and protein interactions. Even at 37 °C, where Cr(III) ligand exchange is thought to occur four to five times fasters than at ambient temperature, pepsin activity was found to remain constant, irrespective of Cr(III) concentration (up to 1000 ppm). It is thought that in the presence of oxidising-reducing agents at physiological temperature, reactions of Cr(III) involving the exchange of ligands may be possible, resulting in toxicity (Nieboer and Jusys, 1988). However, no evidence for this was obtained in this study.

Similarly, an explanation for the results obtained for the observed enzyme activity in the presence of Cr(VI) can be advanced (see Figure 3.12). Pepsin was inhibited at concentrations greater than 100 ppm, whereas collagenase showed no decline in activity to 1000 ppm.

Cr(VI) in phosphate buffer would most likely have consisted of the CrO_4^{2-} ion, which, although presumed capable of oxidising low molecular weight reductants (cysteine, ascorbate, etc.), would be kinetically nonlabile in this reductant free system (Nieboer and Jusys, 1988). Inhibition of pepsin action by Cr(VI) (pH 2, ion as $HCrO_4^-$) at concentrations greater than 100 ppm is consistent with the finding that the $HCrO_4^-$ ion is more reactive (Connett and Wetterhahn, 1983; Nieboer and Jusys, 1988). The hydroxyl group is readily replaced via the formation of an ester with –OH and –SH containing species. As $HCrO_4^-$ contributes only 3 % of the total available Cr at pH 7.4, results observed in this study (see section 3.4.3) are entirely consistent with this view.

Taken together, these findings suggest that the Cr(III) component of leather is unavailable for reaction during enzymatic degradation with collagenase and pepsin. This would also suggest that microbial degradation of chromium tanned leather is not hindered by the reported toxicity of Cr(III) to enzymes

possibly responsible for *in situ* decomposition (Brown *et al.*, 1996). It was assumed that the hydrolysed fragments after enzyme action could be assimilated and utilized by microbial decomposer populations. Barriers to microbial decomposition may result from unknown chromium toxicity to other physiological processes, possibly involving the intracellular conversion of Cr(III) to Cr(VI) or the binding of Cr(III) to microbial DNA.

4.3. Chromium chemistry

Many soils worldwide contain naturally high levels of chromium, with levels ranging from 1 ppm to in excess of 1000 ppm (Barnhart, 1997). Chromium predominantly exists as Cr(III) within soils, and is often bound in crystalline forms such as chromite ($FeCr_2O_4$). Serpentite bedrock is thought to be the source of available chromium in the environment (Gasser *et al.*, 1995; Lottermoser, 1997).

Soils with naturally high levels of chromium are not considered polluted but 'naturally and geochemically' enriched, whereas chromium from anthropogenic sources are considered pollutants, requiring possible remediation (Lottermoser, 1997).

Anthropogenic sources of Cr(III) in the soil result from the disposal of leather waste in landfill, the use of leather based fertilizers and feedstuffs, and the application of sewage sludges onto land.

Previous studies into the *in situ* conversion of Cr(III) to Cr(VI) have shown that oxidation is unlikely in soil systems (Bartlett and Kimble, 1976). However, more recent studies have shown that the presence of oxidised manganese in fresh moist soils may result in the favourable oxidation of Cr(III) to Cr(VI), when the soil pH is greater than 5.5 (Bartlett and James, 1979). It was speculated that the airdrying of soils in previous studies had reduced

manganese oxides to Mn(II), preventing chromium oxidation (Bartlett and James, 1979).

Anthropogenic sources of Cr(III) in the soil are more likely to be oxidised to Cr(VI), as higher levels of available chromium generally exists in added waste. Soils containing high levels of organic matter will be oxidised by the small amount of Cr(VI) formed in the soil, reducing the chromium back to Cr(III) and stabilizing it onto soil particles, where it becomes inert.

In both the Cass and Ilam soil microcosms and the mixed substrate decomposition experiments in this study (see Figure 3.2 and 3.7), formation of Cr(VI) and toxicity to degradation processes may have been suppressed by the quantity of organic matter either as the soil, or litter. Consistent with this, was the observation that decomposition in microcosms containing ignited sand, a lag period was occurred prior to leather decomposition. With high pH (*ca.* 8.5) and the small amount of manganese in the leather substrate (leather ash contained 1.7 % Mn), oxidation of Cr(III) to Cr(VI) may have occurred. Therefore, the lag period could reflect an increased period of time whilst microbial tolerance to the chromium was selected for.

A scenario such as this would have implications for the long term disposal of Cr(III) containing waste in landfill and the general disposal of Cr(III) waste products. Following addition of Cr(III) waste to soil systems, immobilization of the chromium onto the soil matrix is likely to occur, if the soil is high in organic matter. Over time, these soils may weather, increasing the availability of the chromium, or alternately the chromium may become bound within mineral structures, increasing the residence time indefinitely.

The availability of chromium has been correlated with plant growth toxicity, to varying degrees of success. Although a number of studies report chlorosis of plant material and stunting of growth after the incorporation of chromium in soil systems, there are no studies where the toxicity is conclusively attributed to Cr(III). Many of the studies in which Cr(III) has been found to be toxic neglect to mention the effect of pH on chromium (Myttenaere and

Mousny, 1974; Pettersson, 1976), and in cases where leather meal fertilizers have been utilized (at levels of 10-15 % w/w), have failed to account for the probable toxicity of high salt levels (Grubinger *et al.*, 1994). Other reports on the use of 20 % (w/w) leather meal fertilizers (Ciavatta and Sequi, 1989; Govi *et al.*, 1996) suggest that their use harbours no short term (1 year) problems, with Cr released during humification, precipitating in insoluble forms.

In this study, the effect of soluble Cr salts (1000 ppm) on the germination and radicle growth of cucumber seeds showed that both Cr(VI) salts tested were highly inhibitory, and that Cr(III) nitrates and chlorides were moderately inhibitory. Chromium sulphate was found to have no detrimental effect on seed germination or growth. Pigmentation and stunting of radicles in young cucumber seedlings suggested that the toxicity of Cr(VI) was a result of oxidative damage. Cr(III) salt toxicity was thought to result from inhibitory concentrations of nitrate and chloride causing chlorosis of young shoots.

At this time, not enough is known about the long term effects of the continual application of chromium waste to definitively say whether current methods of leather waste disposal are inadequate for future concerns.

4.4. Conclusions

Decomposition of chromium tanned leather as measured by nitrogen mineralization generally increased over time. Increased mineralization resulted from increases in temperature and moisture availability, with maximal rates of decomposition observed at temperatures exceeding ambient. The mean residence time for leather in field soil conditions is likely to be in the order of years. This is contrary to decomposition models of substrates with low C:N ratios, which are generally considered to be of high resource quality.

The incorporation of chromium and pesticides within the collagen matrix are major contributing factors to the lower than expected resource quality of the leather.

Microbial degradation was reduced by the unavailability of accessible carbon and nitrogen within the leather. This was shown by unchanging levels of nitrogen mineralization after amendment of the leather with available carbon and nitrogen. Carbon and nitrogen immobilization data was unfortunately unavailable in this study, as equipment limitations and time restrictions prevented their determination.

Autoclaving caused chemical hydrolysis and denatured the leather, resulting in the loss of collagen structure and increased solubility. Microbial decomposition of hydrolysed samples was rapid after an initial period of minimal activity.

Solubilization of leather with the cumulative action of collagenase and pepsin and assays of enzyme activity in the presence of Cr(III) / Cr(VI), suggested that Cr(III) is nontoxic to proteolytic enzymes when bound and inert within the leather matrix, but may be toxic to physiological functions when intracellularly available to prokaryotic microorganisms.

Decomposition of leather in the field will result in a gradual increase of soil chromium. Maintenance of high levels of soil organic matter in Cr(III) treated soils will result in the unavailability of added chromium. Oxidation of Cr(III) to Cr(VI) is unlikely to be an issue in well aerated acidic soils.

There is minimal evidence for the toxicity of Cr(III) to plant and soil biochemistry at this time, although scope exists for possible toxicity to soil microorganisms through interference of intracellular biochemistry. The effects of Cr(III) on the soil microfauna are unknown. Thus, at this time, the disposal of leather waste into landfill or soils as fertilizer is unlikely to pose any serious environmental threat, although further research into the possible toxicity of Cr(III) containing waste to soil microflora and microfauna is suggested.

4.5. Problems encountered

In decomposition experiments utilizing more than one substrate, such as leather decomposition in soil, or mixed leather / litter decomposition, the extent of mineralization of each component was unquantifiable. In cases where no mineralization was detected, analysis with electron microscopy of microbial growth and substrate degradation, was used as an indicator of decomposition. For optimal analysis, the use of cold stage electron microscopy is recommended.

4.6. Suggested future work

Further investigation into the toxicity of anthropogenic Cr(III) is required, as it's effects on the intracellular physiology of soil microorganisms is unknown. The incomplete removal of pesticides and it's effect of microbial decomposition requires further study, as it was shown in this study, that they may be detrimental to long term decomposition.

The degradation of leather waste in long term field studies needs to be studied. An investigation utilizing litter bags buried in the soil may provide information on the residence time of the waste under natural conditions, and the interactions of soil microflora and microfauna with the leather.

Immobilization data for carbon / nitrogen in microcosm experiments would ideally have been included in this study, but limitations in equipment and time made this impractical. Further studies combining mineralization and immobilization data would allow for a more definitive model of the processes involved in chromium tanned leather decomposition.

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REFERENCES

Aislabie, J. and Loutit, M. (1986). Accumulation of Cr(III) by bacteria isolated from polluted sediment. *Marine Environmental Research* **20**: 221-232.

Anderson, J. (1973). The breakdown and decomposition of sweet chestnut (*Castanea sativa* Mill.) and beech (*Nothofagus sylvatica* L.) leaf litter in two deciduous woodland soils. I. Breakdown, leaching and decomposition. *Oecologica* **12**: 251-274.

Barnhart, J. (1997). Chromium chemistry and implications for environmental fate and toxicity. *Journal of Soil Contamination* **6**: 561-568.

Bartlett, R. and James, B. (1979). Behavior of chromium in soils: III. Oxidation. *Journal of Environmental Quality* **8**: 31-35.

Bartlett, R. and Kimble, J. (1976). Behavior of chromium in soils: I. Trivalent forms. *Journal of Environmental Quality* **5**: 379-383.

Birbir, M. and Ilgaz, A. (1996). Isolation and identification of bacteria adversely affecting hide and leather quality. *Journal of the Society of Leather Technologists and Chemists* **80**: 147-153.

Bremner, J. (1965). Inorganic forms of nitrogen. In *Methods of Soil Analysis Part 2. Chemical and Microbiological Properties.* Monograph #9. C. Black, Ed. American Society of Agronomy, Inc., Madison. pp.1179-1237

Brown, E., Dudley, R. and Elsetinow, A. (1997a). A conformational study of collagen as affected by tanning procedures. *Journal of the American Leather Chemists Association* **92**: 225-233.

Brown, E., King, G. and Chen, J. (1997b). Model of the helical portion of a type I collagen microfibril. *Journal of the American Leather Chemists Association* **92**: 1-7.

Brown, E., Taylor, M. and Marmer, W. (1996). Production and potential uses of co-products from solid tannery waste. *Journal of the American Leather Chemists Association* **91**: 270-276.

Cabeza, L., Taylor, M., Brown, E. and Marmer, W. (1997). Influence of pepsin and trypsin on chemical and physical properties of isolated gelatin from chrome shavings. *Journal of the American Leather Chemists Association* **92**: 200-207.

Cawston, T. and Murphy, G. (1981). Mammalian collagenase. In *Methods in Enzymology.* v.80. L. Lorand, Ed. Academic Press, Inc., New York. pp.711-722

Chakraborty, R. and Sarkar, S. (1998). Enzyme hydrolysis of solid tannery wastes: solid state enzyme production. *Journal of the Society of Leather Technologists and Chemists* **82**: 56-58.

Ciavatta, C. and Sequi, P. (1989). Evaluation of chromium release during the decomposition of leather meal fertilizers applied to the soil. *Fertilizer Reseach* **19**: 7-11.

Clarholm, M. and Rosswall, T. (1980). Biomass and turnover of bacteria in a forest soil and peat. *Soil Biology and Biochemistry* **12**: 49-57.

Collins, Y. and Stotzky, G. (1989). Factors affecting the toxicity of heavy metals to microbes. In *Metal ions and bacteria.* T. Beveridge and R. Doyle, Eds. John Wiley & Sons, Inc., New York. pp.31-90

Connett, P. and Wetterhahn, K. (1983). Metabolism of the carcinogen chromate by cellular constituents. In *Structure and Bonding. Inorganic Elements in Biochemistry.* v.54. Springer-Verlag, Berlin. pp.93-124

DasGupta, S. (1996). Personal Communication. Leather & Shoe Research Association, Palmerston North, New Zeland

Datta, S. and Chandra, A. (1982). Bacterial degradation of leather wastes. In *Waste treatment and utilization, theory and practice of waste management.* v.2. Pergamon Press, Oxford. pp.325-327

de Laune, R., Reddy, C. and Patrick Jr, W. (1981). Organic matter decomposition in soil as influenced by pH and redox conditions. *Soil Biology and Biochemistry* **13**: 533-534.

de Neve, S. and Hofman, G. (1996). Modelling N mineralization of vegetable crop residues during laboratory incubations. *Soil Biology and Biochemistry* **28**: 1451-1457.

De, S. and Chandra, A. (1979). Degradation of organic nitrogenous wastes by a soil streptomycete. *Folia Microbiologica* **24**: 473-477.

Dickinson, C. (1974). Decomposition of litter in soil. In *Biology of plant litter decomposition.* v.2. C. Dickinson and G. Pugh, Eds. Academic Press, London. pp.633-658

El Boushy, A., van der Poel, A., Koene, J. and Dieleman, S. (1991). Tanning waste by-product from cattle hide, its suitability as a feedstuff. *Bioresource Technology* **35**: 321-323.

Fersht, A. (1985). *Enzyme structure and mechanism.* 2nd Edn. WH Freeman and Company, New York

Ford, T. and Mitchell, R. (1992). Microbial transport of toxic metals. In *Environmental Microbiology.* R. Mitchell, Ed. Wiley-Liss, Inc., New York. pp.83-101

Fred, E. and Waksman, S. (1928). *Laboratory Manual of General Microbiology.* 1st Edn. McGraw-Hill Book Company, Inc., . 44-45p.

Frostegård, A., Tunlid, A. and Bååth, E. (1996). Changes in microbial community structure during long-term incubation in two soils experimentally contaminated with metals. *Soil Biology and Biochemistry* **28**: 55-63.

Gasser, U., Juchler, S., Hobson, W. and Sticher, H. (1995). The fate of chromium and nickel in subalpine soils derived from serpentinite. *Canadian Journal of Soil Science* **75**: 187-195.

Gayatri, R., Rajaram, A., Rajaram, R., Govindaraju, K., Rao, J., Nair, B. and Ramasami, T. (1997). Chromium induced structural changes in biomolecules. *Proceedings of the Indian Academy of Sciences (Chemical Sciences)* **109**: 307-317.

Gibbons, R. and MacDonald, J. (1961). Degradation of collagenous substrates by *Bacteriodes melanimogenicus. Journal of Bacteriology* **81**: 614-621.

Glasgow, R. (1997). Personal Communication. The Tasman Tanning Company Ltd, New Zealand

Govi, M., Ciavatta, C., Sitti, L., Bonoretti, G. and Gessa, C. (1996). Influence of leather meal fertilizer on soil organic matter: a laboratory study. *Fertilizer Reseach* **44**: 65-72.

Greenfield, L. (1997). Personal Communication. Department of Plant and Microbial Sciences, University of Canterbury, New Zealand

Grubinger, V., Gutenmann, W., Doss, G., Rutzke, M. and Lisk, D. (1994). Chromium in Swiss chard grown on soil amended with tannery meal fertilizer. *Chemosphere* **28**: 717-720.

Gustavson, K. (1949). Some protein-chemical aspects of tanning processes. In *Advances in protein chemistry*. v.5. M. Anson, J. Edsall and K. Bailey, Eds. Academic Press, Inc, New York. pp.353-421

Halligan, G. (1975). *Leather as a potting mix additive*. Palmerston North, New Zealand Department of Scientific and Industrial Research.

Halligan, G. (1998). Personal Communication. Buckman Laboratories, Palmerston North, New Zealand

Hesse, P. (1971). Carbon and organic matter. In *A textbook of soil chemical analysis*. John Murray Publishers, London. pp.204-254

Jones, J. and Richards, B. (1978). Fungal development and the transformation of ^{15}N-labelled amino- and ammonium nitrogen in forest soils under several management regimes. *Soil Biology and Biochemistry* **10**: 161-168.

Katz, S. and Salem, H. (1994). *The biological and environmental chemistry of chromium*. VCH Publishers, Inc., New York. 214p.

Kennedy, L. (1996). Personal Communication. Buckman Laboratories, New Zealand

Killham, K. (1994). *Soil Ecology*. The Press Syndicate of the University of Cambridge, Cambridge. 242p.

Kono, T. (1968). Purification and partial characterization of collagenolytic enzymes from *Clostridium histolyticum*. *Biochemistry* **7**: 1106-1114.

Kortenkamp, A., Casadevall, M. and da Cruz Fresco, P. (1996). The reductive conversion of the carcinogen chromium (VI) and its role in the formation of DNA lesions. *Annals of Clinical and Laboratory Science* **26**: 160-175.

Lahouti, M. and Peterson, P. (1979). Chromium accumulation and distribution in crop plants. *Journal of the Science of Food and Agriculture* **30**: 136-142.

Lottermoser, B. (1997). Natural enrichment of topsoils with chromium and other heavy metals, Port Macquarie, New South Wales, Australia. *Australian Journal of Soil Research* **35**: 1165-1176.

Mann, B. (1971). Pollution and the leather industry. In *Environment and Industry*. J. Coxon and J. Hogan, Eds. NZ Institute of Chemistry (Inc.). pp.73-76

McGrath, S. (1982). The uptake and translocation of tin and hexavalent chromium and effects on the growth of oat in flowing nutrient solution and in soil. *New Phytologist* **92**: 381-390.

Muthusubramanian, L., Mitra, R. and Sundara Rao, V. (1998). 2-(thiocyanomethylthio)-benzothiazole fungicide on leather: a review. *Journal of the Society of Leather Technologists and Chemists* **82**: 22-23.

Myttenaere, C. and Mousny, J. (1974). The distribution of chromium 51 in lowland rice in relation to the chemical form and to the amount of stable chromium in the nutrient solution. *Plant and Soil* **41**: 65-72.

Nicolardot, B., Fauvet, G. and Cheneby, D. (1994). Carbon and nitrogen cycling through soil microbial biomass at various temperatures. *Soil Biology and Biochemistry* **26**: 253-261.

Nieboer, E. and Jusys, A. (1988). Biological chemistry of chromium. In *Chromium in the natural and human environments*. J. Nriagu and E. Nieboer, Eds. John Wiley & Sons, New York. pp.21-79

Nykvist, N. (1961). Leaching and decomposition of litter. I. Experiments on leaf litter of *Fraxinus excelsior*. *Oikos* **10**: 190-211.

Okada, S., Suzuki, M. and Ohba, H. (1983). Enhancement of ribonucleic acid synthesis by chromium(III) in mouse liver. *Journal of Inorganic Biochemistry* **19**: 95-103.

Paranas, H. (1975). Model for decomposition of organic material by microorganisms. *Soil Biology and Biochemistry* **7**: 161-169.

Peterson, P. and Girling, C. (1981). Other trace metals: chromium. In *Effect of heavy metal on pollution on plants*. v.1. N. Lepp, Ed. Applied Science Publishers, London. pp.222-229

Pettersson, O. (1976). Heavy metal ion uptake by plants from nutrient solutions with metal ion, plant species, and growth period variations. *Plant and Soil* **45**: 445-459.

Pettit, D. and Abbott, S. (1975). Biodeterioration of footwear. In *Microbial aspects of the deterioration of materials*. R. Gilbert and D. Lovelock, Eds. Academic Press, London

Raju, A., Rose, C. and Rao, N. (1997). Enzymatic hydrolysis of tannery fleshings using chicken intestine proteases. *Animal Feed Science Technology* **66**: 139-147.

Richards, B. (1987). *The Microbiology of Terrestrial Ecosystems*. Longman Scientific & Technical, Singapore. 399p.

Rutland, F. (1991). *Environmental compatibility of chromium-containing tannery and other leather product wastes at land disposal sites*. Cincinnati, Leather Industries Research Laboratory, University of Cincinnati.

Sanderson, C. (1982). Applications of ^{51}chromium in cell biology and medicine. In *Biological and environmental aspects of chromium*. S. Langård, Ed. Elsevier Biomedical Press, Amsterdam. pp.101-116

Scott, S. (1992). *The regulation of the emission of contaminants in New Zealand: The past, present and future?* Victoria University, Wellington. 62p. (LLB(Hons) Thesis, Law Faculty)

Seifter, S. and Gallop, P. (1962). Collagenase from *Clostridium histolyticum*. In *Methods in Enzymology*. v.5. S. Colowick and N. Kaplan, Eds. Academic Press, Inc., New York. pp.659-665

Seifter, S. and Harper, E. (1970). Collagenases. In *Methods in Enzymology*. v.19. G. Perlman and L. Lorand, Eds. Academic Press, Inc., New York. pp.613-635

Sharma, D., Chatterjee, C. and Sharma, C. (1995). Chromium accumulation and its effects on wheat (*Triticum aestivum* L. cv. HD 2204) metabolism. *Plant Science* **111**: 145-151.

Shingleton, W., Hodges, D., Brick, P. and Cawston, T. (1996). Collagenase: a key enzyme in collagen turnover. *Biochemistry & Cell Biology* **74**: 759-775.

Shumilla, J., Wetterhahn, K. and Barchowsky, A. (1998). Inhibition of NF-κB binding to DNA by chromium, cadmium, mercury, zinc, and arsenite *in vitro*: evidence of a thiol mechanism. *Archives of Biochemistry and Biophysics* **349**: 356-362.

Sivaparvathi, M., Suseela, K. and Nandy, S. (1986a). Hydrolytic action of *Pseudomonas aeruginosa* on chrome shavings. *Leather Science* **33**: 8-11.

Sivaparvathi, M., Suseela, K. and Nandy, S. (1986b). Purification and properties of *Pseudomonas aeruginosa* protease causing hydrolysis of chrome shavings. *Leather Science* **33**: 303-307.

Skeffington, R., Shewry, P. and Peterson, P. (1976). Chromium uptake and transport in barley seedlings (*Hordeum vulgare* L.). *Planta* **132**: 209-214.

Skooglund, J. (1924). Utilization of hair and leather in the manufacture of commercial fertilizers. *Journal of the American Leather Chemists Association* **19**: 11-14.

Snow, E. (1994). Effects of chromium on DNA replication *in vitro*. *Environmental Health Perspectives* **102**: 41-44.

Stearns, D., Belbruno, J. and Wetterhahn, K. (1995). A prediction of chromium(III) accumulation in humans from chromium dietary supplements. *FASEB Journal* **9**: 1650-1657.

Swift, M., Heal, O. and Anderson, J. (1979). *Decomposition in Terrestrial Ecosystems.* Blackwell Scientific Publications, Oxford. 372p.

Taylor, M., Diefendorf, E., Brown, E. and Marmer, W. (1992). Characterization of products isolated by enzyme treatment of chromium-containing leather waste. *Journal of the American Leather Chemists Association* **87**: 380-388.

Taylor, M., Diefendorf, E., Thompson, C., Brown, E., Marmer, W. and Cabeza, L. (1997). Extraction of value added byproducts from the treatment of chromium containing collagenous leather industry waste. *Journal of the Society of Leather Technologists and Chemists* **81**: 5-13.

Tietema, A. and van Dam, D. (1996). Calculating microbial carbon and nitrogen transformations in acid forest litter with ^{15}N enrichment and dynamic simulation modelling. *Soil Biology and Biochemistry* **28**: 953-965.

Tristram, G. (1949). Amino acid composition of purified proteins. In *Advances in Protein Chemistry*. v.5. M. Anson, J. Edsall and K. Bailey, Eds. Academic Press, Inc., New York

Wackett, L., Orme-Johnson, W. and Walsh, C. (1989). Transition metal enzymes in bacterial metabolism. In *Metal ions and bacteria*. T. Beveridge and R. Doyle, Eds. John Wiley & Sons, Inc., New York. pp.165-206

Waksman, S. (1952). Decomposition of plant and animal residues in soil and compost. In *Soil Microbiology*. Wiley, New York. pp.95-123

Waldvogel, F. and Swartz, M. (1969). Collagenolytic activity of bacteria. *Journal of Bacteriology* 98: 662-667.

Walker, D. (1994). *Microbial decomposition of flower litter.* University of Canterbury, Christchurch. 102p. (MSc Thesis, Department of Plant and Microbial Sciences)

Watkins, N. and Barraclough, D. (1996). Gross rates of N mineralization associated with the decomposition of plant residues. *Soil Biology and Biochemistry* 28: 169-175.

Welton, R. and Woods, D. (1973). Halotolerant collagenolytic activity of *Achromobacter iophagus*. *Journal of General Microbiology* 75: 191-196.

Williams, S. and Gray, T. (1974). Decomposition of litter on the soil surface. In *Biology of plant litter decomposition*. v.2. C. Dickinson and G. Pugh, Eds. Academic Press, London. pp.611-632

Windholz, M., Ed. (1976). *The Merck Index.* 9th Edn. Merck & Co, Inc., Rahway

Appendices

A. Preparation of media

A.1. PDA media

39.0 g of Potato dextrose agar (Difco) was made up to 1000 ml with distilled water and autoclaved at 121 °C for 20 minutes. Media pH was 5.5.

A.2. NA media

8.0 g of Nutrient broth (Merck) and 15.0 g of bacteriological agar (Difco) was made up to 1000 ml with distilled water and autoclaved at 121 °C for 20 minutes. Media pH was 6.9.

A.3. Preparation of Cr ash supplemented media

Oven-dried unwashed leather shavings were ignited in an electric muffle furnace (570 °C, 8 hours), and ground with mortar and pestle when cool. This was added to NA and PDA media to give 100 to 300 ppm Cr ash, prior to autoclaving. pHs of 300 ppm ash supplemented media were 6.8 for NA and 5.6 for PDA.

Elemental analysis of ash via SEM, showed the composition of the ash to be : 72.6 % Cr, 11.1 % S, 6.7 % Na, 6.6 % Ca, 1.7 % Mn, and 1.3 % Mg.

B. Crone's nitrogen-free stock powder

Potassium chloride (KCl)	10.0 g
Calcium sulphate ($CaSO_4.2H_2O$)	2.5 g
Magnesium sulphate ($MgSO_4.7H_2O$)	2.5 g
Tricalcium phosphate ($Ca_3(PO_4)_2$)	2.5 g
Ferric phosphate ($FePO_4$)	2.5 g

Salts are mixed and ground to a fine powder. A stock solution of 1.5 $g.l^{-1}$ of the powder is made up in ddH_2O. (Fred and Waksman, 1928)

C. Autoclaved leather decomposition – 30 day results.

Comparison of results for 30 day decomposition of autoclaved leather. Experiment was performed twice.

Autoclaved leather type	Mean N-mineralization	Standard error	Final pH
Unwashed shavings	47.6	21.0	8.4
Unwashed ground shavings	63.4	6.6	8.4
Unwashed shavings	47.1	16.5	8.4
Unwashed ground shavings	64.0	4.3	8.5

D. Amino acid composition of purified collagen and gelatin.

Amino acid	Collagen amino acid composition (%)	Gelatin amino acid composition (%)
Arginine	8.59	8.55
Histidine	0.74	0.73
Lysine	4.47	4.60
Glutamic acid	11.3	11.2
Aspartic acid	6.3	5.6
Amide NH_3	0.1	0.09
Glycine	27.2	26.9
Alanine	9.5	9.3
Valine	3.4	3.3
Leucine / Isoleucine	5.6	5.23
Phenylalanine	2.5	2.55
Proline	15.1	14.8
Tryptophan	-	-
Serine	3.37	3.18
Threonine	2.28	2.2
Cystine / Cysteine	-	-
Hydroxyproline	14.0	14.5
Hydroxylysine	1.1	1.2
Methionine	0.8	0.9

Taken from Tristram (1949).